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(54) Title: INHIBITING CARDIOMYOCYTE DEATH

(57) Abstract

The invention features methods of inhibiting cardiomyocyte death in a mammal by administering to the myocardium of the mammal a heme oxygenase (HO). Methods of preserving an organ for transplantation and methods of inhibiting vascular stenosis are also within the invention.

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INHIBITING CARDIOMYOCYTE DEATH

Related Application Information

This application claims priority from provisional application no. 60/121,946, filed on February 25, 1999, and provisional application no. 60/098,377, filed on August 28, 1998.

Statement as to Federally Sponsored Research

This invention was made with U.S. Government support under National Institutes of Health grants RO1 GM53249, KO8 HL03274, and KO8 HL03194. The government has certain rights in the invention.

Background of the Invention

The invention relates to treatment of cardiovascular disease.

Myocardial infarction is one of the most common diagnoses of hospitalized patients in western countries. In the United States, over 1.5 million myocardial infarctions occur annually, and mortality from acute myocardial infarction is approximately 25 per cent. Thrombolytic therapy and reperfusion of ischemic myocardium, e.g., using percutaneous transluminal coronary angioplasty (PTCA), have decreased mortality rates among patients who have suffered an acute myocardial infarction. Nevertheless, myocardial damage and heart failure resulting from a failure to achieve early revascularization or from reperfusion injury remain a significant clinical problem.

Summary of the Invention

The invention features methods of minimizing myocardial damage by salvaging hypoxic myocardial tissue before it becomes irreversibly injured. For example, a method of inhibiting cardiomyocyte death in a mammal, e.g., a human, who has suffered a myocardial infarction or who has myocarditis is carried out by locally

administering to the myocardium of the mammal a heme oxygenase (HO) polypeptide. Preferably, the HO polypeptide has the amino acid sequence of a naturally-occurring heme oxygenase-1 (HO-1), heme oxygenase-2 (HO-2), or heme oxygenase-3 (HO-3), or a biologically active fragment thereof.

Compositions, such as hemin, hemoglobin, or heavy metals, e.g., tin or nickel, that increase production of endogenous HO, are also administered to inhibit cardiomyocyte death or damage. For example, overexpression of HO-1 is induced in vascular cells by exposure to heme, heavy metals, endotoxin and hyperoxia, hyperthermia, shear stress and strain, UV light, or reactive oxygen species. By "overexpression" is meant a level of protein production that at least 20% greater than that present in the tissue under normal physiologic conditions. Preferably, the level of HO-1 expression in vascular tissue in the presence of an inducing agent is at least 20% greater than that in the absence of the inducing agent; more preferably, the level of expression is at least 50% greater, more preferably, the level of expression is at least 100% greater, and most preferably, the level of expression is at least 200% greater than that in the absence of an inducing agent. Inhibition of cardiomyocyte death is also achieved by locally administering to the myocardium of a mammal a DNA encoding a HO. HO expression by target cell, e.g., VSMC, is increased by administering to the cells exogenous DNA encoding HO, e.g., a plasmid containing DNA encoding human HO-1 or HO-2 under the control of a strong constitutive promoter. Oxidative stress leads to cell death by apoptosis and/or necrosis. By neutralizing reactive oxygen species, HO reduces cardiomyocyte damage and death due to oxidative stress.

The invention also includes a method of inhibiting cardiomyocyte death *in vitro* by contacting cardiomyocytes with an HO or DNA encoding an HO. For example, a method of preserving isolated myocardial tissue, e.g., a donor heart to be used for transplantation, is carried out by bathing or perfusing the tissue with a solution containing an HO or a DNA encoding an HO. The method allows prolonged storage of organs after removal from the donor and prior to transplantation into a recipient by reducing irreversible ischemic tissue damage. By "isolated myocardial tissue" is meant tissue that has been removed from a living or recently deceased mammal. Preferably, a donor heart is preserved in an HO solution for 0.5-6 hours prior to transplantation. More preferably, the organ is preserved for greater than 6 hours, e.g., 8, 10, 12, and up to 24 hours.

A method of inhibiting vascular stenosis or restenosis in a mammal, e.g., a human, is also within the invention. The method is carried out by locally administering to the site of a vascular injury or a site which is at risk of developing a stenotic lesion a compound which inhibits expression of HO-1, and as a result, VSMC proliferation. To inhibit vascular stenosis or restenosis (which occurs at a relatively late stage after the occurrence of a vascular injury), the compound is administered at least one month after an injury such as surgery or angioplasty. For example, such treatment is administered 3 weeks to several months (e.g., 2 months or 3 months) post-injury. Preferably, the compound inhibits transcription of the gene encoding HO-1 or inhibits translation of HO-1 mRNA into an HO-1 polypeptide in a vascular cell, e.g., a vascular smooth muscle cell (VSMC), of the mammal. The vascular cell is preferably an aortic smooth muscle cell, e.g., an aortic smooth muscle cell located in the region of an artery

affected by vascular stenosis or restenosis such as the site of balloon angioplasty or coronary bypass surgery. For example, transforming growth factor-β1 (TGF-β1) is administered to inhibit production of HO-1 mRNA and HO gene product.

For antisense therapy, the compound is a antisense nucleic acid molecule containing at least 10 nucleotides, the sequence of which is complementary to an mRNA encoding all or part of a wild type HO polypeptide. Preferably, the compound, e.g., an antisense oligonucleotide or antisense RNA produced from an antisense template, inhibits HO expression. The antisense nucleic acid inhibits HO expression by inhibiting translation of HO mRNA. For example, antisense therapy is carried out by administering a single stranded nucleic acid complementary at least a portion of HO mRNA to interfere with the translation of mRNA into protein, thus reducing the amount of functional HO produced in the cell. The method includes the step of identifying a mammal having undesired vascular stenosis or restenosis or at risk of developing such a condition. For example, the mammal to be treated is one who needs or has recently undergone PTCA, coronary artery bypass surgery, other vascular injury, that stimulates vascular smooth muscle cell proliferation that results in undesired vascular stenosis or restenosis.

For treatment of a vascular injury soon after the injury or stress has occurred, an HO polypeptide, e.g., HO-1, or a nucleic acid encoding an HO polypeptide, is administered to a mammal within minutes until approximately one week post-injury. Augmentation of the level of HO in injured vascular tissue shortly after the injury has occurred inhibits an initial increase in local VSMC proliferation post-injury. For example, such early

stage intervention is carried out within 24 hours post-injury.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

The drawings will first be briefly described.

Drawings

Fig. 1 is a diagram of the targeted gene disruption strategy used in making an HO-1-deficient mouse.

Fig. 2 is a bar graph showing that hypoxia increases hematocrit in HO-1 +/+ and -/- mice.

Fig. 3 is a bar graph showing that hypoxia markedly increases ventricular weight in HO-1 -/- mice.

Fig. 4 is a diagram of the mouse model of vein graft stenosis.

Fig. 5A is a line graph showing luminal occlusion of an artery into which a vein patch has been grafted.

Fig. 5B is a diagram of a vein graft.

Fig. 6 is a diagram of plasmid containing a myosin heavy chain promoter which directs cardiospecific expression of a polypeptide-encoding DNA to which it is operably linked.

Fig. 7 is a bar graph showing that chronic hypoxia increases right ventricular systolic pressure. Wild type (+/+) and HO-1-deficient (-/-) mice were exposed to normoxia or chronic hypoxia (10% oxygen). Right ventricular pressure was measured under normoxic conditions (open bars) or after five weeks of hypoxia (filled bars). Error bars indicate standard deviation. *$P<0.05$ vs. animals exposed to normoxia within the same group ($n = 5$ in each group).

Fig. 8 is a bar graph showing that HO-1 -/- arterial smooth muscle cells are more sensitive to

oxidative stress compared to wild type smooth muscle cells.

Fig. 9 is an autoradiograph of a Northern blot assay showing expression of a human HO-1 (hHO-1) transgene in a transgenic mouse.

Fig. 10 is an autoradiograph of a Western blot showing the presence of a hHO-1 gene product in tissues of HO-1 transgenic mice.

HO-1-deficient mice

HO-1-deficient (HO-1-/-) mice were produced using a standard targeted gene deletion strategy to delete exon 3 (Fig. 1). The murine HO-1 gene contains 5 exons and 4 introns, spanning approximately 7 kilobases (kb). The targeting construct was made by deleting the largest exon (exon 3) which contains 492 nucleotides out of the 867 nucleotides of the entire open reading frame. This deletion renders the HO-1 enzyme non-functional. An *Xho*I/*BamH*I fragment of the *neo* cassette from pMC1*neo* PolyA plasmid was subcloned into pBluescript II SK (Stratagene, La Jolla, CA) to generate pBS-*neo*. To generate pBS-*neo*-HO-1, the 3 kb *Xho*I fragment of the HO-1 gene spanning from exon 1 to the end of intron 2 was subcloned into the *Xho*I site of pBS-*neo* in the same orientation as the *neo* cassette. The 4 kb HO-1 *BamH*I-*EcoR*I fragment containing a small portion of intron 3, exon 4, and exon 5 was subcloned into *BamH*I and *EcoR*I site of pPGK-TK to generate pPGK-TK-HO-1. The 7 kb *BamH*I-*Cla*I fragment (filled in with Klenow) from pPGK-TK-HO-1 was then subcloned into *BamH*I and *Xba*I sites (filled in with Klenow) sites of pBS-*neo*-HO-1 to generate the HO-1 targeting construct. The linearized targeting construct was transfected into murine D3 embryonic stem (ES) cells, and a clone with the correct homologous recombination (yielding the appropriately disrupted HO-1 gene) injected into blastocysts and used to generate HO-1

deficient mice. The survival rate of HO-1 -/- mice was 25% of the expected survival rate, and the mice were grossly normal. The mice were deficient in HO-1 mRNA and HO-1 protein but not HO-2 mRNA or protein.

HO-1 transgenic mice

Standard techniques were used to generate mice which express a hHO-1 transgene in heart tissue. The transgene was cloned under the control of the cardiac α-myosin heavy chain promoter for expression preferentially in cardiovascular tissue. One group of transgenic mice were engineered to express hHO-1 DNA in the sense orientation, and another group expressed hHO-1 DNA in the antisense orientation. As shown in Fig. 9, hHO-1 mRNA was detected in the heart (ventricle) of the transgenic mouse but not in other tissues tested. Western blot analysis confirmed the presence of a transgenic hHO-1 gene product in heart tissue (ventricles) of hHO-1 transgenic mice. hHO-1 transgenic mice are used to evaluate the effect of HO-1 expression (and overexpression) in cardiovascular tissue, e.g., in response to injury or stress.

Inhibition of cardiomyocyte death

Oxidative stress caused by such conditions as ischemia and reperfusion injury induces myocardial dysfunction and cardiomyocyte death. HO is an enzyme that catalyzes oxidation of heme to generate carbon monoxide (CO; which can increase cellular cGMP) and biliverdin (which is a potent antioxidant). HO-1 is an inducible isoform of HO, whereas HO-2 is constitutively expressed. Expression of HO-1 is induced in the cardiovascular system by such stimuli as hypoxia, hyperoxia, cytokines such as interleukin-1β (IL-1β), endotoxemia, heat shock, and ischemia. HO-1 also regulates VSMC growth. Expression of inducible HO (HO-1)

is markedly induced in the cardiovascular system by stimuli such as increased pressure and hypoxia.

To study the effect of HO-1 on mammalian responses to hypoxia such as that manifested in clinical conditions, e.g., high altitude pulmonary edema, myocardial infarction, myocarditis, pulmonary hypertension, pulmonary embolism, pulmonary valve stenosis, congenital heart disease, and chronic obstructive pulmonary disease (e.g., emphysema), mice were subjected to chronic hypoxia. In accordance with a standard model of pulmonary hypertension, mice were kept in a 10% O_2 chamber for 7 weeks. Two groups (Group I = five HO-1 +/+ mice; Group II = five HO-1 -/- mice) were studied. Two of the HO-1 deficient mice died at week 7; none of the HO-1 +/+ mice died. As shown in Fig. 2, exposure of the mice to hypoxic conditions resulted in an increase in hematocrit in both wild type and knockout (HO-1 -/-) mice, indicating a high level of tissue hypoxia of the mice. Under normoxia conditions, the heart weight of HO-1-deficient and wild type mice was comparable, but under hypoxic conditions the ventricular weight of HO-1-deficient mice greater than that of the HO-1-deficient mice kept under normoxic conditions (Fig. 3). For example, exposure to hypoxia for 7 weeks caused a 32% increase in the ventricular weight index in wild type mice, whereas in HO-1-deficient mice, the ventricular weight index after 7 weeks of hypoxia increased 100% (compared to normoxic wild type mice. Changes in the ventricular weight reflected mainly a right ventricular effect, as the RV(LV+septum) increased in HO-1-deficient mice compared to wild type mice exposed to hypoxia for 7 weeks.

Fig. 7 shows the effect of hypoxia on right ventricular systolic pressure, an indicator of pulmonary arterial systolic pressure. Right ventricular systolic

pressure in wild type and HO-1 -/- mice did not differ under normoxic conditions (P = 0.80; Fig. 7, open bars). Although five weeks of hypoxia increased right ventricular systolic pressure, it did so to a similar degree in wild type and HO-1 -/- mice (P = 0.43; Fig. 7, filled bars).

In HO-1-deficient mice, exposure to conditions of chronic hypoxia resulted in more dramatic hypertrophy and dilation of the right ventricle of the heart compared to that observed in wild type mice. Evidence of massive cardiomyocyte death was detected and large organized thrombi attached to areas of infarct were also detected in HO-1-deficient mice but not in wild type mice.

When stained with Masson's trichrome stain (which detects collagen), an increase in collagen fibers was observed in tissue sections of the right ventricle of HO-1 -/- mice in response to hypoxia compared to the amount of collagen detected in wild type sections. These data indicate evidence of scar formation and repair mechanisms in the hearts of HO-1-deficient mice under hypoxic conditions. Tissue sections were also stained with PECAM stain (which detects endothelial cells). Increased blood vessel formation was detected in the right ventricles of HO-1 -/- mice in response to hypoxia compared to wild type mice. These data suggest that H0-1 inhibits angiogenesis in response to hypoxia.

The mechanism of cardiomyocyte death in HO-1 -/- mice under hypoxic conditions was evaluated by histological analysis, immunocytochemistry, and TdT-mediated dUTP-biotin nickend labeling (TUNEL assay). The standard TUNEL assay detects apoptosis. Ventricles were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or Masson's trichrome. To detect oxidation-specific lipid-protein adducts, heart tissue

sections were immunostained with polyclonal antibody MAL-2 (anti-malondialdehyde-lysine; Rosenfeld et al., 1990, Arteriosclerosis 10:336-349) and counterstained with methyl green. TUNEL was used to detect DNA breaks in apoptotic cells *in situ*. Red staining in the nuclei of the cells indicated a positive reaction; the cells were also counterstained with methyl green. Pulmonary vascular remodeling was assessed in lungs that had been perfused with saline through the pulmonary artery and fixed with 4% paraformaldehyde instilled through the trachea. Muscularization of peripheral vessels was determined using standard methods, e.g., that described in Klinger et al., 1993, J. Appl. Physiol. 75:198-205. The nuclei of cardiomyocytes of HO-1-deficient mice subjected to hypoxic conditions stained positive, providing evidence that apoptosis contributes to the mechanism of death of cardiomyocytes under these conditions.

Additional histological analyses were undertaken to confirm that chronic hypoxia induces right ventricular infarction in HO-1-deficient mice. Cardiomyocytes were intact in ventricular sections from wild type mice exposed to 7 weeks of hypoxia, but ventricular sections from HO-1-deficient mice exposed to 7 weeks of hypoxia showed mononuclear inflammatory cell infiltration, extensive cardiomyocyte degeneration, and death with focal calcification. These observations indicate that infarcts were 1-2 weeks old. The right ventricular infarcts did not appear to result from vascular occlusion, because the coronary arteries supplying blood to the right ventricle were patent in H0-1-deficient mice.

To detect collagen accumulation indicative of fibrosis, ventricular sections were stained with Masson's trichrome. After 7 weeks of hypoxia, cells surrounding

blood vessels stained positive for collagen in hearts from wild type mice. In hearts from HO-1-deficient mice exposed to hypoxia for 7 weeks, early collagen deposition was present throughout the lesion. The degree of fibrosis was consistent with early scar formation and a lesion of 1-2 weeks old.

Two out of the six hearts from 7-week hypoxic HO-1-deficient mice did not exhibit right ventricular infarcts; however, close examination of the hearts revealed focal areas of myocardial degeneration without evidence of extensive inflammatory cell infiltration. These hearts showed early evidence of myocardial damage. Heart from wild type and HO-1-deficient mice examined after 5 weeks of hypoxia displayed no cardiomyocyte degeneration or death and no extensive mononuclear inflammatory cell infiltration. Cells surrounding blood vessels stained positive for collagen in hearts from wild type and HO-1-deficient mice housed for 5 weeks under normoxic and hypoxic conditions. In contrast with the right ventricular free walls from HO-1-deficient mice exposed to 7 weeks of hypoxia (which showed deposition of collagen), no collagen deposition was evident in the right ventricular free walls from HO-1-deficient mice exposed to 5 weeks of hypoxia. These data confirm that in the HO-1-deficient mice exposed to 7 weeks of hypoxia, myocardial infarcts were less than 2 weeks old.

To assess oxidative damage in hearts with infarcts taken from HO-1-deficient mice, ventricular tissue sections were immunostained with MAL-2 which detects oxidation-specific lipid-protein adducts. In contrast to the minimal MAL-2 staining observed in hearts from wild type mice, MAL-2 staining was intense in cells (predominantly cardiomyocytes) beneath the infarcted area of the right ventricle in HO-1-deficient mice. These data indicate the presence of severe oxidative damage

within and around the infarct site. No TUNEL-positive cardiomyocytes were detectable in right ventricles from wild-type mice, but a significant number of TUNEL-positive cells surrounded the infarct site in right ventricles form HO-1-deficient mice.

The data described herein indicate that (1) pulmonary vascular remodeling in response to hypoxia is similar in HO-1 +/+ and -/- mice, (2) hypoxia induces more severe right ventricular hypertrophy in HO-1 -/-mice than in HO+/+ mice, and (3) in HO-1 -/- (but not +/+ mice), massive cardiomyocyte death occurs with large organized thrombi attached to the infarct site. Although some cardiomyocyte death appears to be due to necrosis, apoptosis is a significant mechanism of cardiomyocyte death. Hypoxia and elevated pulmonary arterial pressure increase cardiac production of reactive oxygen species, which play a significant role in myocardial death during ischemia/reperfusion.

Although myocardial infarction has been shown to increase oxidative stress, a 2-3 fold increase in the nitration of protein tyrosine residues (which indicates the presence of the potent oxidant peroxynitrite) was detected in noninfarcted HO-1-deficient hearts exposed to 7 weeks of hypoxia. These data indicate that an increase in oxidative stress precedes gross myocardial infarction.

Evidence of extensive lipid peroxidation in the zone of right ventricular infarction supports the conclusion that the absence of HO-1 in cardiomyocytes leads to an accumulation of reactive oxygen species that causes cardiomyocyte death. Administration of HO-1 or overexpression of HO-1 protects against cardiomyocyte damage from hemodynamic stress or ischemia/reperfusion.

Adaptation of the cardiovascular system to hypoxia

The gene deletion studies described herein indicate that HO-1 plays an important protective role *in*

vivo in the adaptation of the cardiovascular system to hypoxia. Right ventricles from HO-1 -/- mice were severely dilated and contained right ventricular infarcts with mural thrombi.

Humans and animals respond to hypoxia by exhibiting pulmonary vascular remodeling, pulmonary hypertension, and hypertrophy of the right ventricle. The data described herein were obtained using a mouse model of vascular injury which mimics the human response. Hypoxia induces HO-1 expression in the lung, and CO generated by hypoxic VSMCs inhibits proliferation of these cells.

The data described herein indicate that the absence of HO-1 results in a maladaptive response in cardiomyocytes exposed to hypoxia-induced pulmonary hypertension. In the absence of HO-1, VSMC are more sensitive to oxidative stress and have a maladaptive response to pressure overload. HO-1 has a protective effect on cardiomyocytes and VSMC subjected to stress such as pressure-induced injury and secondary oxidative damage.

Therapeutic administration of HO

In the absence of HO-1, cardiomyocytes undergo apoptotic cell death when subjected to stress such as pressure overload or exposure to reactive oxygen species and that death can be inhibited by contacting the cells with HO. For example, HO-1, HO-2, or HO-3 protein or polypeptide (or DNA encoding HO-1, HO-2, or HO-3) is administered locally to heart tissue affected by hypoxic conditions. One means for accomplishing local delivery is providing an HO or DNA encoding and HO on a surface of a vascular catheter, e.g., a balloon catheter coated with an antioxidant, which contacts the wall of the blood vessel to deliver therapeutic compositions at the site of

contact. Drug delivery catheters can also be used to administer solutions of therapeutic compositions.

HO-1 is therapeutically overexpressed (e.g., by administering an inducing agent to increase expression from the endogenous gene) or by administering DNA (alone or in a plasmid) encoding an HO such as HO-1 or HO-2 (or an active fragment thereof, i.e., a fragment has the activity of inhibiting cardiomyocyte death). Inducing agents that stimulate HO-1 expression in cells include hemin, hemoglobin, and heavy metals, e.g., $SnCl_2$ or $NiCl_2$. For example, 250 mmol/kg of body weight of $SnCl_2$ or $NiCl_2$ is administered subcutaneously or 15 mg/kg of body weight of hemin is administered intraperitoneally to laboratory animals. Doses for human patients are determined and optimized using standard methods.

Tables 1 and 3 show human HO-1 and HO-2 cDNA, respectively, in which the polypeptide-encoding nucleotides are designated in bold type and the termination codon is underlined. Tables 2 and 4 show the amino acid sequences of human HO-1 and HO-2, respectively. Tables 5 and 6 show the nucleotide and amino acid sequence of rat HO-3.

TABLE 1: Human HO-1 cDNA

1 tcaacgcctg cctcccctcg agcgtcctca gcgcagccgc cgcccgcgga gccagcacga

61 acgagcccag caccggccgg **atggagcgtc cgcaacccga cagcatgccc caggatttgt**

121 cagaggccct gaaggaggcc accaaggagg tgcacaccca ggcagagaat gctgagttca

181 tgaggaactt tcagaagggc caggtgaccc gagacggctt caagctggtg atggcctccc

241 tgtaccacat ctatgtggcc ctggaggagg agattgagcg caacaaggag agcccagtct

301 tcgcccctgt ctacttccca gaagagctgc accgcaaggc tgccctggag caggacctgg

361 ccttctggta cgggccccgc tggcaggagg tcatccccta cacaccagcc atgcagcgct

421 atgtgaagcg gctccacgag gtggggcgca cagagcccga gctgctggtg gcccacgcct

481 acacccgcta cctgggtgac ctgtctgggg gccaggtgct caaaaagatt gcccagaaag

541 ccctggacct gcccagctct ggcgagggcc tggccttctt caccttcccc aacattgcca

601 gtgccaccaa gttcaagcag ctctaccgct cccgcatgaa ctccctggag atgactcccg

661 cagtcaggca gagggtgata gaagaggcca agactgcgtt cctgctcaac atccagctct

721 ttgaggagtt gcaggagctg ctgacccatg acaccaagga ccagagcccc tcacgggcac

781 cagggcttcg ccagcgggcc agcaacaaag tgcaagattc tgcccccgtg gagactccca

841 gagggaagcc cccactcaac acccgctccc aggctccgct tctccgatgg gtccttacac

901 tcagctttct ggtggcgaca gttgctgtag ggctttatgc catgtgaatg caggcatgct

961 ggctcccagg gccatgaact ttgtccggtg gaaggccttc tttctagaga gggaattctc
1021 ttggctggct tccttaccgt gggcactgaa ggctttcagg gcctccagcc ctctcactgt
1081 gtccctctct ctggaaagga ggaaggagcc tatggcatct tccccaacga aaagcacatc
1141 caggcaatgg cctaaacttc agaggggggcg aaggggtcag ccctgccctt cagcatcctc
1201 agttcctgca gcagagcctg gaagacaccc taatgtggca gctgtctcaa acctccaaaa
1261 gccctgagtt tcaagtatcc ttgttgacac ggccatgacc actttccccg tgggccatgg
1321 caatttttac acaaacctga aaagatgttg tgtcttgtgt ttttgtctta ttttttgttgg
1381 agccactctg ttcctggctc agcctcaaat gcagtatttt tgttgtgttc tgttgttttt
1441 atagcagggt tggggtggtt tttgagccat gcgtgggtgg ggagggaggt gtttaacggc
1501 actgtggcct tggtctaact tttgtgtgaa ataataaaca acattgtctg
(SEQ ID NO:1)

Table 2: Human HO-1 amino acid sequence

MERPQPDSMP QDLSEALKEA TKEVHTQAEN AEFMRNFQKG QVTRDGFKLV MASLYHIYVA
LEEEIERNKE SPVFAPVYFP EELHRKAALE QDLAFWYGPR WQEVIPYTPA MQRYVKRLHE
VGRTEPELLV AHAYTRYLGD LSGGQVLKKI AQKALDLPSS GEGLAFFTFP NIASATKFKQ
LYRSRMNSLE MTPAVRQRVI EEAKTAFLLN IQLFEELQEL LTHDTKDQSP SRAPGLRQRA
SNKVQDSAPV ETPRGKPPLN TRSQAPLLRW VLTLSFLVAT VAVGLYAM (SEQ ID NO:2)

Table 3: Human HO-2 cDNA

1 gggctgactg gaggctggcg gacaggcgac agacctgcgg caggaccaga ggagcgagac

61 gagcaagaac cacacccagc agcaatgtca gcggaagtgg aaacctcaga gggggtagac

121 gagtcagaaa aaaagaactc tggggcccta gaaaaggaga accaaatgag aatggctgac

181 ctctcagagc tcctgaagga agggaccaag gaagcacacg accgggcaga aaacacccag

241 tttgtcaagg acttcttgaa aggcaacatt aagaaggagc tgtttaagct ggccaccacg

301 gcactttact tcacatactc agccctcgag gaggaaatgg agcgcaacaa ggaccatcca

361 gcctttgccc ctttgtactt ccccatggag ctgcaccgga aggaggcgct gaccaaggac

421 atggagtatt tctttggtga aaactgggag gagcaggtgc agtgccccaa ggctgcccag

481 aagtacgtgg agcggatcca ctacataggg cagaacgagc cggagctact ggtggcccat

541 gcatacaccc gctacatggg ggatctctcg gggggccagg tgctgaagaa ggtggcccag

601 cgagcactga aactccccag cacaggggaa gggacccagt tctacctgtt tgagaatgtg

661 gacaatgccc agcagttcaa gcagctctac cgggccagga tgaacgccct ggacctgaac

721 atgaagacca aagagaggat cgtggaggcc aacaaggctt ttgagtataa catgcagata

781 ttcaatgaac tggaccaggc cggctccaca ctggccagag agaccttgga ggatgggttc

841 cctgtacacg atgggaaagg agacatgcgt aaatgccctt tctacgctgc tgaacaagac

901 aaagggctgg agggcagcct gtcccttccg acaagctatg ctgtgctgag gaagcccagc

961 ctccagttca tcctggccgc tggtgtggcc ctagctgctg gactcttggc ctggtactac

1021 atgtgaagca cccatcatgc cacaccggta ccctcctccc gactgaccac tggcctaccc

1081 ctttctccag ccctgactaa actaccacct caggtgactt tttaaaaaat gctgggttta

1141 agaaaggcaa ccaataaaag agatgctaga gcctcgtctg acagcatcct ctctatgggc

1201 catattccgc actgggcaca ggccgtcacc ctgggagcag tcggcacagt gcagcaagcc

1261 tggcccccga cccagctcta ctccaggctt ccacacttct gggccctagg ctgcttccgg

1321 tagtccctgt ttttgcagta catgggtgac tatctccct gttggaggtg agtggcctgt

1381 aagtccaagc tgtgcgaggg ggccttgctg gatgctgctg tacaacttct gggcctctct

1441 tggaccctgg gagtgagggt gggtgtgggt ggaagcctca gaggccttgg gagctcatcc

1501 ctctcaccca gaatccctct aacccttggg tgcggtttgc tcagccccag cttatctcct

1561 cctccgcctg tgtaaatgct ccagcactca ataaagtggg ctttgcaagc taaaaaaaaa

1621 aaaaaaa (SEQ ID NO:3)

Table 4: Human HO-2 amino acid sequence

MSAEVETSEGVDESEKKNSGALEKENQMRMADLSELLKEGTKEAHDRAENTQFVKDF
LKGNIKKELFKLATTALYFTYSALEEEMERNKDHPAFAPLYFPMELHRKEALTKDME
YFFGENWEEQVQCPKAAQKYVERIHYIGQNEPELLVAHAYTRYMGDLSGGQVLKKVA
QRALKLPSTGEGTQFYLFENVDNAQQFKQLYRARMNALDLNMKTKERIVEANKAFEY
NMQIFNELDQAGSTLARETLEDGFPVHDGKGDMRKCPFYAAEQDKGLEGSLSLPTSY
AVLRKPSLQFILAAGVALAAGLLAWYYM (SEQ ID NO:4)

Table 5: Rat HO-3 nucleotide sequence

1 tttcagggat ttttgcgatt cctctctgta gacttctact
tgttctctaa gggagttctt
61 catgtctttc ttgaagtcat ccagcatcat gatcaaatat
gattttgaaa ctagatcttg
121 cttttctggt gtgtttggat attccatgtt tgttttggtg
ggagaattgg gctccgatga
181 tggcatgtag tcttggtttc tgttgcttgg tttcctgcgc
ttgcctctcg ccatcagatt
241 atctctagtg ttactttgtt ctgctatttc tgacagtggc
tagactgtcc tataagcctg
301 tgtgtcagga gtgctgtaga ccttttttcc tctctttcag
tcagttatgg gacagagtgt
361 tctgcttttg ggcgtgtagt ttttcctctc tacaggtctt
cagctgttcc tgtgggcctg
421 tgtcttgagt tcaccaggca gctttcttgc agcagaaaat
ttggtcatac ctgtgatcct
481 gaggctcaag ttcgctcgtg gggtgctgtc caggggctct
ctgcagcggg cacaaccagg
541 aagacctgtg cggccccttc cggagcttca gtgcaccagg
gttccagatg gcctttggcg
601 ttttcctctg gcgtccgaga tgtatgtaca gagagcagtc
tcttctggtt tcccaggctt
661 gtctgcctct ctgaaggttc agctctccct cccacgggat
ttgggtgcag agaactgttt

721 atccggtctg tttctttcag gttccggtgg tgtctcaggc aggtgtcgtt cctgcgccct

781 cccccatggg accagaggcc ttatacagtt tcctcttggg ccagggatgt gggcaggggt

841 gagcagtgtt ggtggtctct tccgtctgca gcctcaggag tgccacctga ccaggcggtt

901 gggtctctct ctgagaattt cattttaaa tcattcatta aaatgtcatg acttgatgtc

961 ctgctgtccg tctcacgccc tcagctgtaa cagtgccgag ggagtcactg aagaagagac

1021 tgaatgacca gagtatgggc agcacagaca actcaacaaa aatgtcttca gaggtggaga

1081 ctgcggaggc cgtagatgag tcagagaaga actctatggc atcagagaag gaaaaccatt

1141 ccaaaatagc agacttttct gatcttctga aggaagggac aaaggaagca gatgaccggg

1201 cagaaaatac ccagtttgtc aaagacttct tgaaaggaaa cattaagaag gagctattta

1261 agctggccac cactgcactt tcatactcag cccctgagga ggaaatggat tcactgacca

1321 aggacatgga gtacttcttt ggtgaaaact gggaggaaaa agtgaagtgc tctgaagctg

1381 cccagacgta tgtggatcag attcactatg tagggcaaaa tgagccagag catctggtgg

1441 cccatactta ctctacttac atgggggaa acctttcagg ggaccaggta ctgaagaagg

1501 agacccagcc ggtccccttc actagggaag ggactcagtt ctacctgttt gagcatgtag

1561 acaatgctaa gcaattcaag ctattctact gcgctagatt gaatgccttg gacctgaatt

1621 tgaagaccaa agagaggatt gtggaggaag ccaccaaagc ctttgaatat aatatgcaga

1681 tattcagtga actggaccag gcaggctcca taccagtaag agaaacccta aagaatgggc

1741 tctcaatact tgatgggaag ggaggtgtat gcaaatgtcc ctttaatgct gctcagccag
1801 acaaaggtac cctgggaggc agcaactgcc ctttccagat gtccatggcc ttgctgagga
1861 agcctaactt gcagctcatt ctagttgcca gtatggcctt ggtagctgga cttttagcct
1921 ggtactacat gtgaagggcc tgtcaagttg tttgcatcct atctcaacat cctaccactt
1981 gttccttccc cacctccacc tctgcctaga actaccacct caggtgacat ttttaatgtt
2041 gggtttgaga aaatgagcaa ccaataaaag acagacccta gaaaaaagtc atgacttaag
2101 tggcacgggg acacctaaag tcacactttg tgcttcagac atactttctt tctctatttc
2161 aacactgaat tcgggaagta acctactact attaataata aatgctacac aatgcataat
2221 aaaaa (SEQ ID NO:5)

Table 6: Rat HO-3 amino acid sequence

MSSEVETAEAVDESEKNSMASEKENHSKIADFSDLLKEGTKEADDRAENTQFVKDFL KGNIKKELFKLATTALSYSAPEEEMDSLTKDMEYFFGENWEEKVKCSEAAQTYVDQI HYVGQNEPEHLVAHTYSTYMGGNLSGDQVLKKETQPVPFTREGTQFYLFEHVDNAKQ FKLFYCARLNALDLNLKTKERIVEEATKAFEYNMQIFSELDQAGSIPVRETLKNGLS ILDGKGGVCKCPFNAAQPDKGTLGGSNCPFQMSMALLRKPNLQLILVASMALVAGLL AWYYM (SEQ ID NO:6)

An HO preferably has an amino acid sequence that is at least 85% identical (preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably at least 100% identical) to the amino acid sequence of SEQ ID NO: 2, 4, or 6. DNA encoding an HO preferably has nucleotide sequence that is at least 50% identical (preferably at least 75%, more preferably at least 85%, more preferably at least 95%, most

preferably at least 100% identical) to the nucleotide sequence of the coding region of SEQ ID NO:1, 3, or 5. The per cent identity of nucleotide and amino acid sequences is determined using the Sequence Analysis Software Package developed by the Genetics Computer Group (University of Wisconsin Biotechnology Center, Madison, WI), employing the default parameters thereof.

To be clinically beneficial, expression of HO from an endogenous gene or expression of recombinant HO from exogenous DNA need not be long term. For example, to inhibit cardiomyocyte death associated with a myocardial infarction or other acute condition which results in oxidative stress to the tissue, the most critical period of treatment is the first three months after injury. Thus, gene therapy to express recombinant HO for even a period of days or weeks after administration of the HO-encoding DNA (which administration is prior to or soon after an injury) minimizes cell death, inhibits VSMC proliferation, and therefore confers a clinical benefit.

For local administration of DNA to cardiovascular tissue, standard gene therapy vectors used. Such vectors include viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO 89/07136; Rosenberg et al., 1990, N. Eng. J. Med. 323(9):570-578), adenovirus (see, e.g., Morsey et al., 1993, J. Cell. Biochem., Supp. 17E,), adeno-associated virus (Kotin et al., 1990, Proc. Natl. Acad. Sci. USA 87:2211-2215,), replication defective herpes simplex viruses (HSV; Lu et al., 1992, Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sept. 22-26, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), and any modified versions of these vectors. The invention may utilize any other delivery system which accomplishes *in vivo* transfer of nucleic acids into eukaryotic cells. For example, the

nucleic acids may be packaged into liposomes, e.g., cationic liposomes (Lipofectin), receptor-mediated delivery systems, non-viral nucleic acid-based vectors, erythrocyte ghosts, or microspheres (e.g., microparticles; see, e.g., U.S. Patent No. 4,789,734; U.S. Patent No. 4,925,673; U.S. Patent No. 3,625,214; Gregoriadis, 1979, Drug Carriers in Biology and Medicine, pp. 287-341 (Academic Press,). Naked DNA may also be administered. Alternatively, a plasmid which directs cardiospecific expression (e.g., a plasmid containing a myosin heavy chain (αMHC) promoter; Fig. 6) of an HO-encoding sequence can be used for gene therapy. Expression of an HO (encoded, e.g., by the coding sequences of SEQ ID NO:1, 3, or 5) from such a constitutive promoter is useful to inhibit cardiomyocyte death *in vivo*. Nucleic acids which hybridize at high stringency to the coding sequences of SEQ ID NO:1, 3, or 5 and which encode a polypeptide which has a biological activity of an HO polypeptide (e.g., inhibition of cardiomyocyte death) are also used for gene therapy for vascular injury. To determine whether a nucleic acid hybridizes to a reference nucleic acid at a given stringency, hybridization is carried out using standard techniques, such as those described in Ausubel *et al.* (*Current Protocols in Molecular Biology*, John Wiley & Sons, 1989). "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration, *e.g.*, wash conditions of 65°C at a salt concentration of approximately 0.1 X SSC. "Low" to "moderate" stringency refers to DNA hybridization and wash conditions characterized by low temperature and high salt concentration, *e.g.*, wash conditions of less than 60°C at a salt concentration of at least 1.0 X SSC. For example, high stringency conditions may include hybridization at

about 42°C, and about 50% formamide; a first wash at about 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% x SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to an CHF-1 gene are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at about 42°C, about 6X SSC, and about 1% SDS; and a second wash at about 50°C, about 6X SSC, and about 1% SDS. To determine whether a polypeptide encoded by a hybridizing nucleic acid has a biological activity of an HO polypeptide, the polypeptide is evaluated using any of the functional assays to measure HO activity described herein, e.g., measuring VSMC proliferation or cardiomyocyte death.

For gene therapy of cardiovascular tissue, fusigenic viral liposome delivery systems known in the art (e.g., hemagglutinating virus of Japan (HVJ) liposomes or Sendai virus-liposomes) are useful for efficiency of plasmid DNA transfer (Dzau et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:11421-11425). Using HVJ-liposomes, genes are expressed from plasmid DNA delivered to target tissues *in vivo* for extended periods of time (e.g., greater than two weeks for heart and arterial tissue and up to several months in other tissues).

DNA for gene therapy can be administered to patients parenterally, e.g., intravenously, subcutaneously, intramuscularly, and intraperitoneally. Sustained release administration such as depot injections or erodible implants, e.g., vascular stents coated with DNA encoding an HO, may also be used. The compounds may also be directly applied during surgery, e.g, bypass surgery, or during angioplasty, e.g, an angioplasty catheter may be coated with DNA encoding an HO. The DNA

is then deposited at the site of angioplasty. DNA or an inducing agent is administered in a pharmaceutically acceptable carrier, i.e., a biologically compatible vehicle which is suitable for administration to an animal e.g., physiological saline. A therapeutically effective amount is an amount which is capable of producing a medically desirable result, e.g., expression of HO, in a treated animal. Such an amount can be determined by one of ordinary skill in the art. As is well known in the medical arts, dosage for any given patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, severity of arteriosclerosis or vascular injury, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for intravenous administration of DNA is approximately 10^6 to 10^{22} copies of the DNA molecule.

HO-based therapy for cardiovascular disorders depends on when (in the course of a vascular injury) the patient is encountered. HO-1 -/- VSMC initially proliferated at a faster rate compared to wild type VSMC within days after an insult. Increasing local HO-1 levels at this stage inhibits VSMC growth and confers a clinical benefit. For example, if the patient is encountered at an early stage (e.g., within one week of cardiovascular stress or injury), the patient is treated by augmenting the local level of HO-1 (e.g., by administering an HO polypeptide, by increasing expression of endogenous HO, or by standard gene therapy techniques described above to produce recombinant HO *in vivo*) to inhibit the growth of VSMC and decrease the size of a myocardial infarct. In contrast, if the patient is encountered at a later stage (e.g., several weeks, 1 month, 2 months, and up to 3 months after an injury), the

patient is treated by inhibiting HO expression to decrease formation of a stenotic lesion, e.g., by antisense therapy, as described below.

Organ and tissue preservation

Concerns about irreversible ischemic tissue damage arise when a donor organ, e.g., a heart, is removed from the donor and stored for more than about 5-6 hours before transplantation into the recipient. *Ex vivo* treatment of a donor organ to reduce tissue damage by inhibiting death of cardiomyocytes is carried out by immersing the organ in a solution containing an inducing agent, an HO, e.g, HO-1 or HO-2, or a nucleic acid encoding an HO prior to transplantation. By "ex vivo treatment" is meant treatment that takes place outside of the body. For example, *ex vivo* treatment is administered to an organ (or a tissue fragment or dissociated cells) that has been removed from a mammal and which will be returned to the same or a different mammal (at the same or different anatomical site) after the treatment. For example, effective DNA delivery to cells in solid organs achieved by contacting the organ with a combination of DNA, liposomes and transferrin during the cold ischemic time prior to transplantation (see, e.g., Hein et al. 1998, Eur. J. Cardiothorac. Surg. 13:460-466). An organ may also be perfused or electroporated with solution containing HO-encoding DNA. Vectors and delivery systems described above for gene therapy applications are suitable for organ preservation and cell preservation *in vitro*.

Inhibition of restenosis

VSMC proliferation contributes to graft stenosis and restenosis following vascular injury such as that resulting from coronary angioplasty and coronary bypass surgery. Patients with restenosis have a significantly

poorer clinical outcome compared to patients without restenosis.

Using a mouse model of vascular graft stenosis in which the stenosis develops rapidly and closely mimics the development of vascular graft stenosis in humans, the effect of HO-1 on VSMC proliferation was examined. A patch of jugular vein was grafted onto a carotid artery in normal and HO-1 deficient mice to create composite vessels that mimic vein grafts used for bypass surgery (Fig. 4). The vein patch is subject to increased pressure which leads to an increase in local VSMC proliferation and occlusion of the blood vessel (Figs. 5A-B). In wild type mice, there was robust formation of a neointima (characterized by proliferating VSMC) in the vein graft. In contrast, tissue sections of the neointima of HO-1 -/- mice revealed a necrotic mass. The HO-1 -/- neointima was a complex lesion characterized by mostly acellular material, indicating death of VSMC. HO-1 -/- VSMC are more susceptible to H_2O_2-induced death compared to VSMC isolated from wild type mice (Fig. 8). These data indicate that HO-1 is required for VSMC proliferation at later stages post-injury and that local inhibition of HO-1 expression, e.g., by antisense therapy, is useful to inhibit graft stenosis or restenosis in patients affected or who at risk of developing such conditions.

The data described herein indicate that (1) in response to increased pressure, VSMC proliferate in the neointima of the venous patch in HO-1 +/+ mice, and (2) in contrast, massive cell death occurs in the neointima of the venous patch in HO-1 -/- mice.

Patients undergoing invasive vascular procedures, e.g., balloon angioplasty, are at risk for developing undesired vascular stenosis or restenosis. Angioplasty, used to treat arteriosclerosis, involves the insertion of

catheters, e.g., balloon catheters, through an occluded region of a blood vessel in order to expand it. However, the aftermath of angioplasty may be problematic. Restenosis, or closing of the vessel, can occur as a consequence of injury, e.g., mechanical abrasion associated with the angioplasty treatment. This restenosis is caused by proliferation of smooth muscle cells stimulated by vascular injury. Other anatomical disruptions or mechanical disturbances of a blood vessel, e.g., laser angioplasty, coronary artery surgery, atherectomy, coronary artery stents, and coronary bypass surgery, may also cause vascular injury and subsequent proliferation of smooth muscle cells.

Therapeutic approaches, such as antisense therapy or ribozyme therapy are used to inhibit HO expression, and as a result, VSMC proliferation that leads to neointimal thickening. The antisense strand (either RNA or DNA) is directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. Alternatively, a vector-containing sequence which, which once within the target cells is transcribed into the appropriate antisense mRNA, may be administered. Nucleic acids complementary to all or part of the HO cDNA (SEQ ID NO: 1, 3, or 5) may be used in methods of antisense treatment to inhibit expression of HO. Antisense treatment is carried out by administering to a mammal, such as a human, DNA containing a promoter, e.g., a cardiospecific promoter, operably linked to a DNA sequence (an antisense template), which is transcribed into an antisense RNA. By "operably linked" is meant that a coding sequence and a regulatory sequence(s) (*i.e.*, a promoter) are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the regulatory sequence(s). Alternatively, as mentioned

above, antisense oligonucleotides may be introduced directly into vascular cells. The antisense oligonucleotide may be a short nucleotide sequence (generally at least 10, preferably at least 14, more preferably at least 20 (e.g., at least 30), and up to 100 or more nucleotides) formulated to be complementary to a portion, e.g., the coding sequence, or all of HO mRNA. Oligonucleotides complementary to various portions of HO-1 or HO-2 mRNA can readily be tested *in vitro* for their ability to decrease production of HO in cells, using standard methods. Sequences which decrease production of HO message in *in vitro* cell-based or cell-free assays can then be tested *in vivo* in rats or mice to determine whether HO expression (or VSMC proliferation) is decreased.

Ribozyme therapy can also be used to inhibit gene expression. Ribozymes bind to specific mRNA and then cut it at a predetermined cleavage point, thereby destroying the transcript. These RNA molecules may be used to inhibit expression of a gene encoding a protein involved in the formation of vein graft stenosis according to methods known in the art (Sullivan et al., 1994, J. Invest. Derm. 103:85S-89S; Czubayko et al., 1994, J. Biol. Chem. 269:21358-21363; Mahieu et al, 1994, Blood 84:3758-65; Kobayashi et al. 1994, Cancer Res. 54:1271-1275).

Standard methods of administering antisense therapy have been described (see, e.g., Melani et al., 1991, Cancer Res. 51:2897-2901). Antisense nucleic acids which hybridize to HO-encoding mRNA can decrease or inhibit production of HO by associating with the normally single-stranded mRNA transcript, thereby interfering with translation and thus, expression of HO. Such nucleic acids are introduced into target cells by standard vectors and/or gene delivery systems such as those

described above for gene therapy. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses and adeno-associated viruses, among others. For example, antisense oligodesoxynucleotides, e.g., oligonucleotides which have been modified to phosphorthioates or phosphoamidates, withstand degradation after delivery and have been successfully used to inhibit gene expression in a model of reperfusion injury (see, e.g., Haller et al., 1998, Kidney Int. 53:1550-1558). Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal: e.g., physiological saline. A therapeutically effective amount of a compound is an amount which is capable of producing a medically desirable result in a treated animal, e.g., inhibition of expression of HO-1 or a decrease in VSMC proliferation.

Compositions that inhibit HO activity, e.g., its role in the promotion of VSMC proliferation, are also administered to inhibit VSMC-mediated stenosis or restenosis. For example, metalloporphyrins, e.g., zinc protoporphyrin IX (ZnPP), zinc mesoporphyrin IX (ZnMP), tin protoporphyrin IX (SnPP), tin mesoporphyrin IX (SnMP), zinc deuteroporphyrin IX 2,4 bis glycol (ZnDPBG), chromium protoporphyrin (CrPP), cobalt protoporphyrin (CoPP), and manganese metalloporphyrin (MnPP) are administered to mammals at μmol/kg doses to inhibit HO activity. SnPP has safely been administered to human infants at doses of 0.5 μmol/kg to 100μmol/kg of body weight. HO-inhibitory doses for local administration are determined using methods known in the art.

Parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal delivery routes, may be used to deliver the compounds that inhibit

HO activity or expression, with local vascular administration being the preferred route. Dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. For antisense therapy, a preferred dosage for administration of nucleic acids is from approximately 10^6 to 10^{22} copies of the nucleic acid molecule. As described above, local administration to a site of vascular injury or to cardiac tissue is accomplished using a catheter or indwelling vascular stent.

Other embodiments are within the following claims.

What is claimed is:

1. A method of inhibiting cardiomyocyte death in a mammal comprising locally administering to the myocardium of said mammal a heme oxygenase (HO).

2. The method of claim 1, wherein said mammal has suffered a myocardial infarction.

3. The method of claim 1, wherein said mammal has myocarditis.

4. The method of claim 1, wherein said HO is heme oxygenase-1 (HO-1).

5. The method of claim 1, wherein said HO is heme oxygenase-2 (HO-2).

6. A method of inhibiting cardiomyocyte death in a mammal comprising locally administering to the myocardium of said mammal a DNA encoding a HO.

7. The method of claim 6, wherein said HO is HO-1.

8. The method of claim 6, wherein said HO is HO-2 or HO-3.

9. A method of inhibiting cardiomyocyte death *in vitro*, comprising contacting cardiomyocytes with an HO.

10. A method of inhibiting cardiomyocyte death *in vitro*, comprising contacting cardiomyocytes with DNA encoding an HO.

11. The method of claim 10, wherein said HO is HO-1.

12. The method of claim 10, wherein said HO is HO-2.

13. A method of preserving isolated myocardial tissue comprising perfusing said tissue with a solution comprising an HO or a DNA encoding an HO.

14. A method of inhibiting vascular restenosis in a mammal comprising locally administering to the site of a vascular injury a compound which inhibits expression of HO-1.

15. The method of claim 14, wherein said compound inhibits HO-1 transcription in a vascular cell of said mammal.

16. The method of claim 15, wherein said vascular cell is an aortic smooth muscle cell.

17. The method of claim 14, wherein said mammal is a human.

18. The method of claim 14, wherein said compound inhibits translation of HO-1 mRNA in a vascular cell of said mammal.

19. The method of claim 18, wherein said compound consists of a single stranded nucleic acid complementary to at least a portion of said HO-1 mRNA.

20. A method of inhibiting vascular restenosis in a mammal comprising identifying a mammal having vascular restenosis or at risk of developing vascular restenosis and administering to said mammal a compound which inhibits expression of HO-1.

21. The method of claim 14, wherein said compound is administered to said mammal at least one month after a vascular injury.

22. The method of claim 14, wherein said compound is administered to said mammal at least two months after a vascular injury.

23. The method of claim 14, wherein said compound is administered to said mammal at least three months after a vascular injury.

24. A method of inhibiting vascular smooth muscle cell proliferation in a mammal comprising administering to an injured vascular tissue of said mammal a HO, wherein said HO is administered within 24 hours after a vascular injury.

25. The method of claim 24, wherein said HO is administered to said mammal for up to one week after a vascular injury.

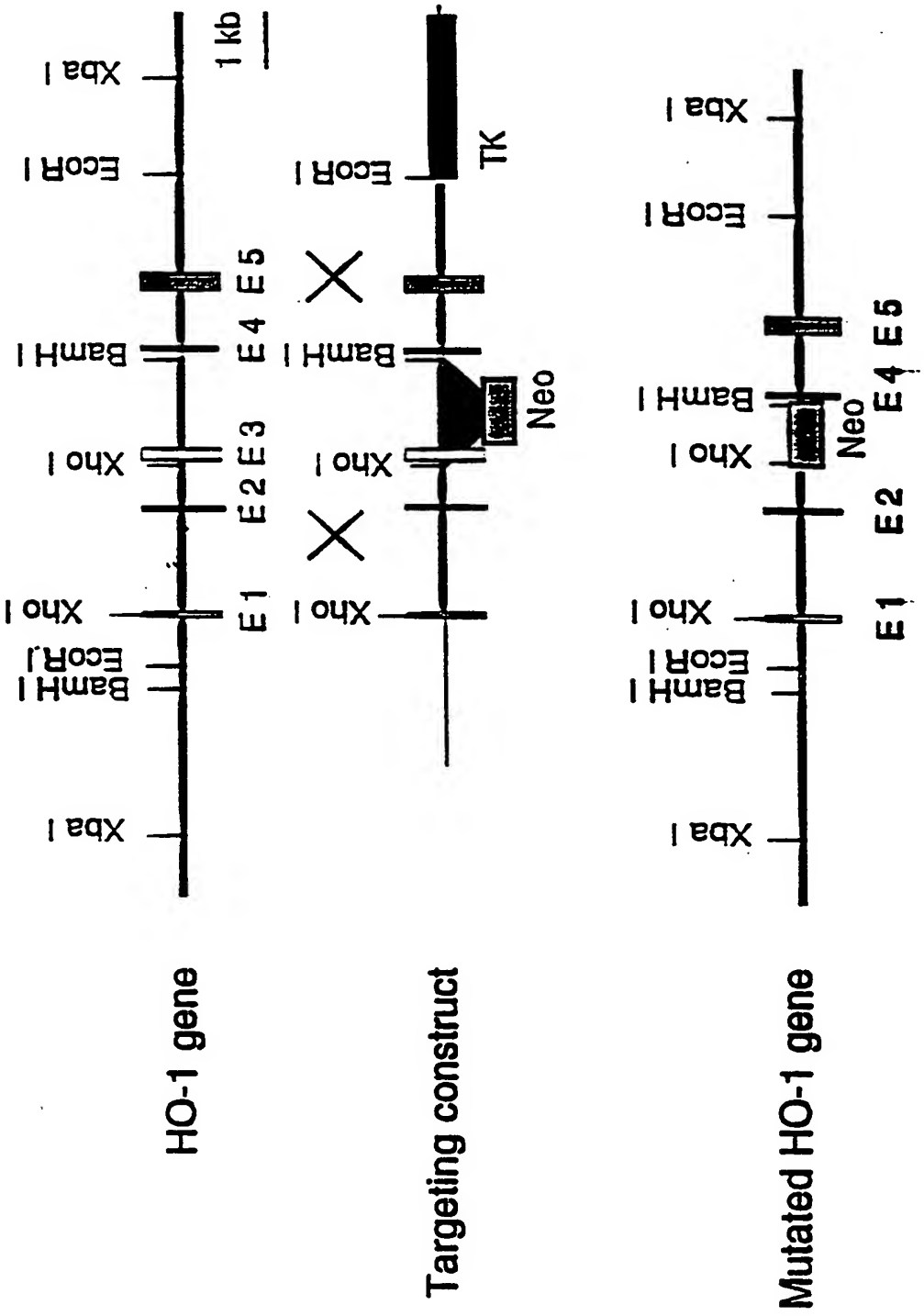

1 kb
Xba I
EcoR I
BamH I
Xho I
E1
E2
E3
E4
E5
HO-1 gene
TK
Neo
Targeting construct
Mutated HO-1 gene

FIG. 1

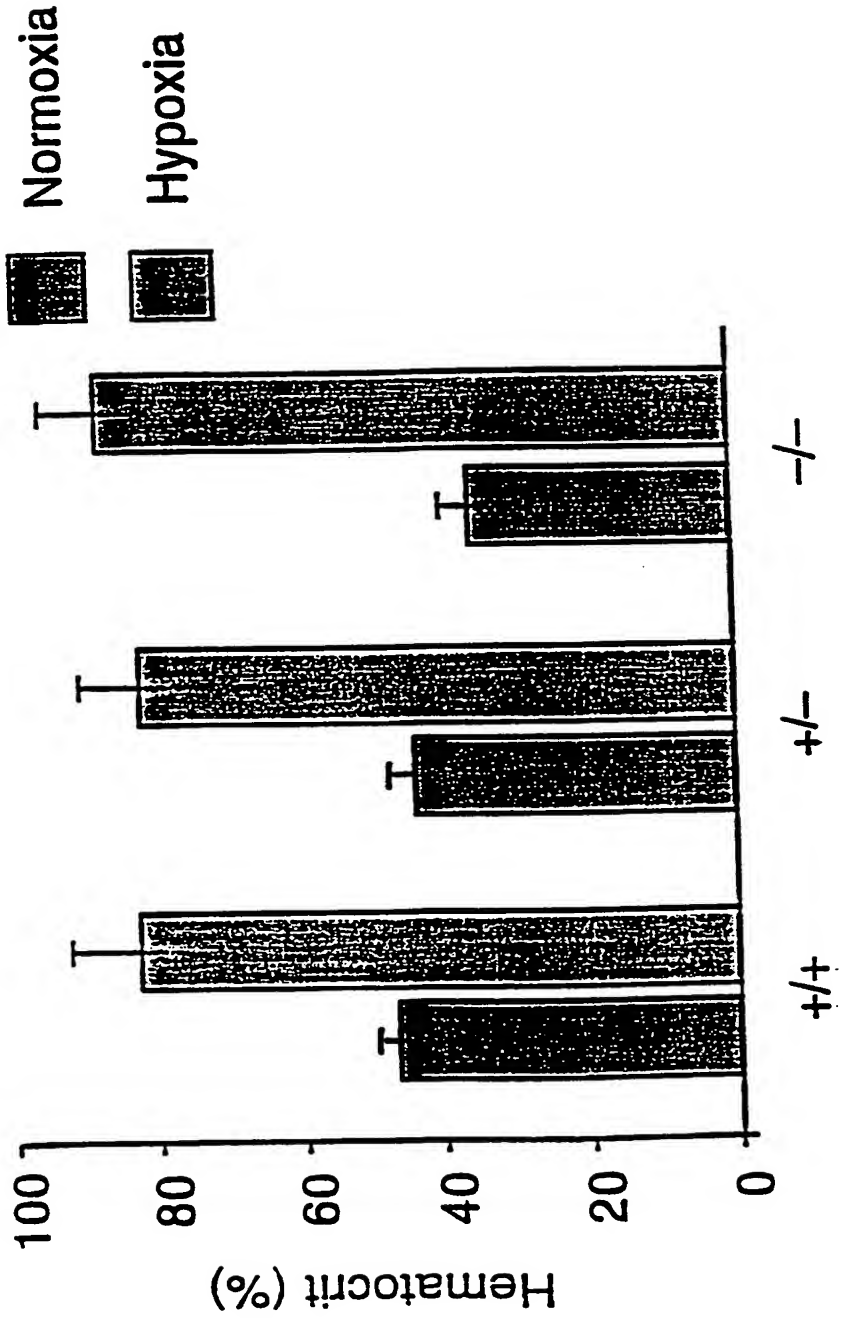


FIG.2

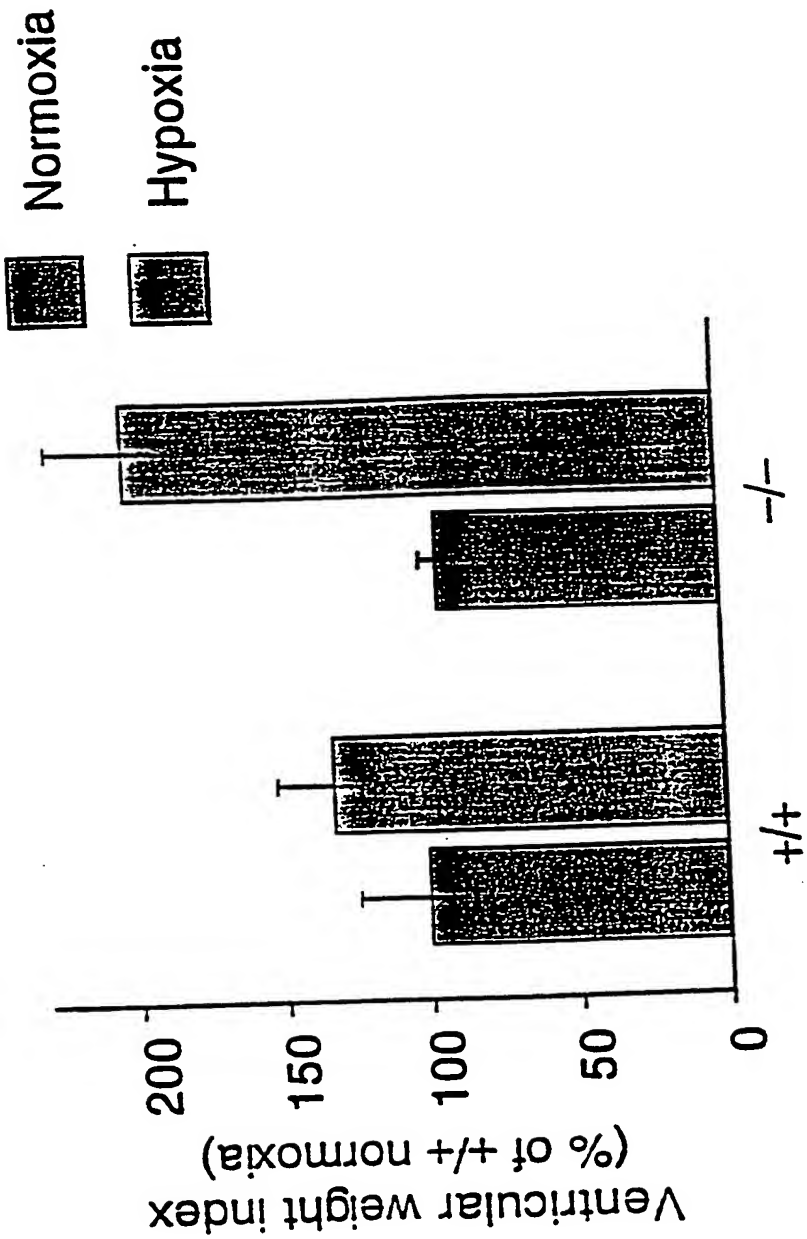

Normoxia
Hypoxia
Ventricular weight index
(% of +/+ normoxia)
200
150
100
50
0
+/+
-/-

FIG. 3

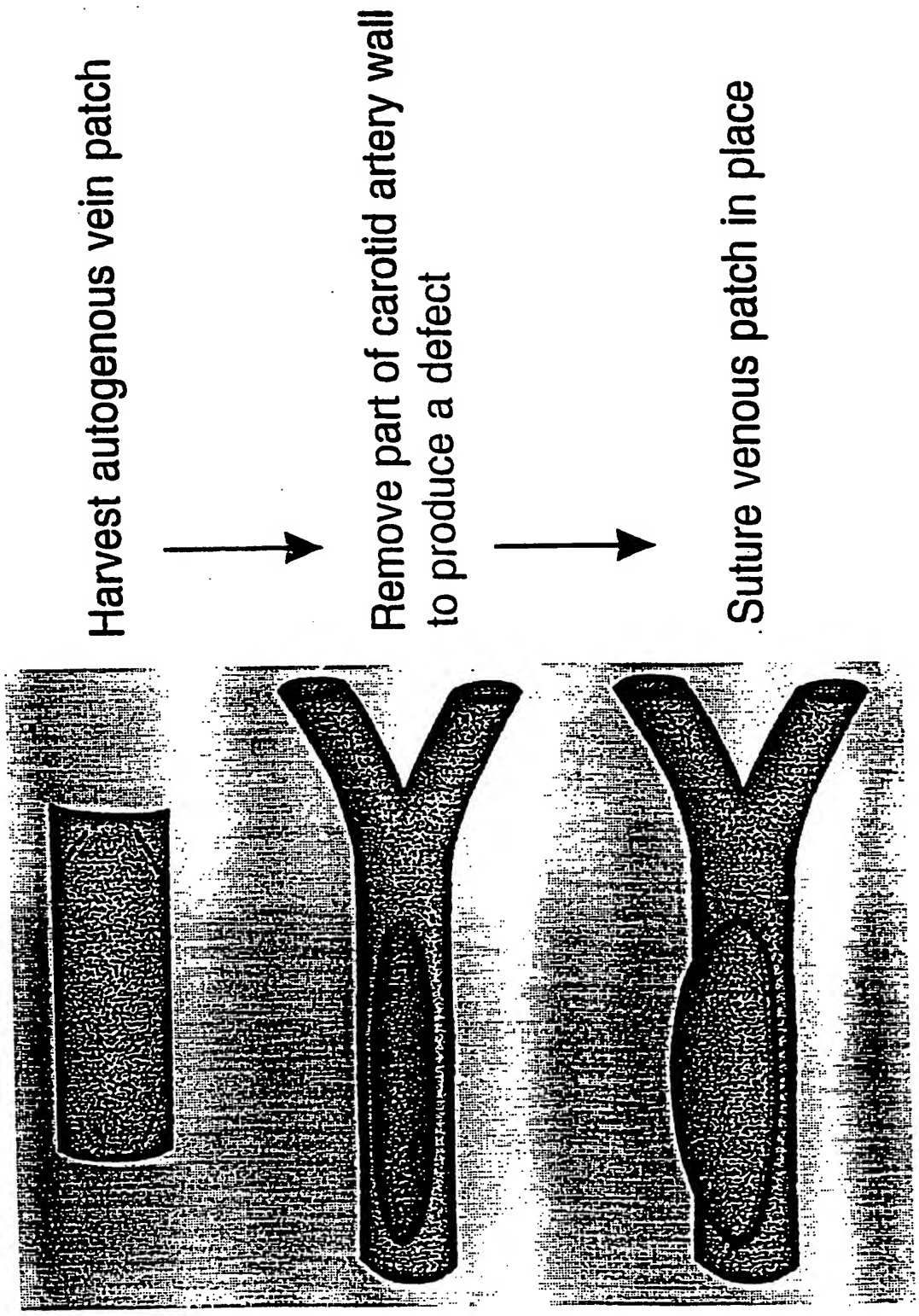

Harvest autogenous vein patch
Remove part of carotid artery wall to produce a defect
Suture venous patch in place

FIG. 4

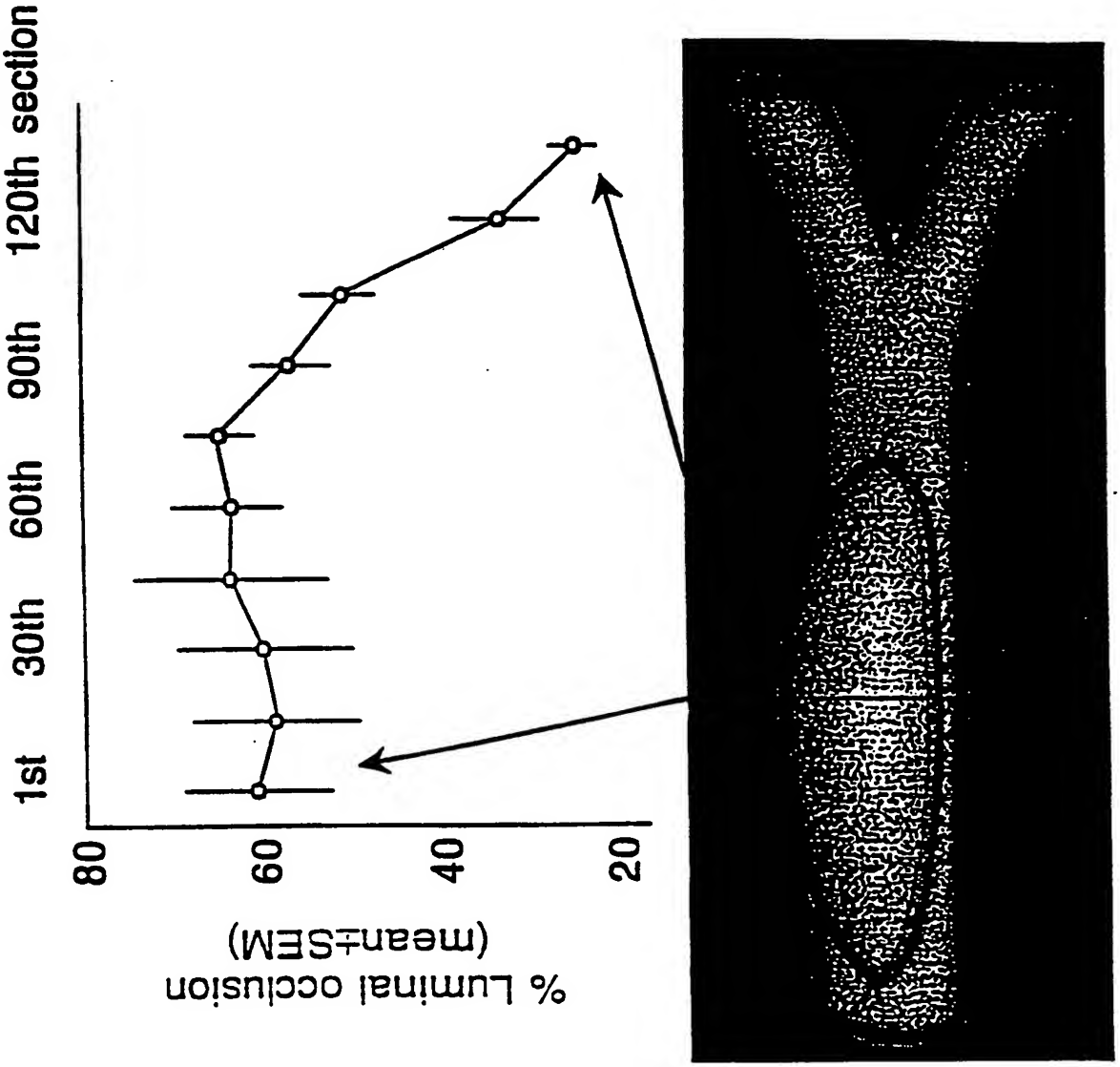

1st
30th
60th
90th
120th section
80
60
40
20
% Luminal occlusion
(mean±SEM)

FIG. 5A

FIG. 5B

FIG. 6

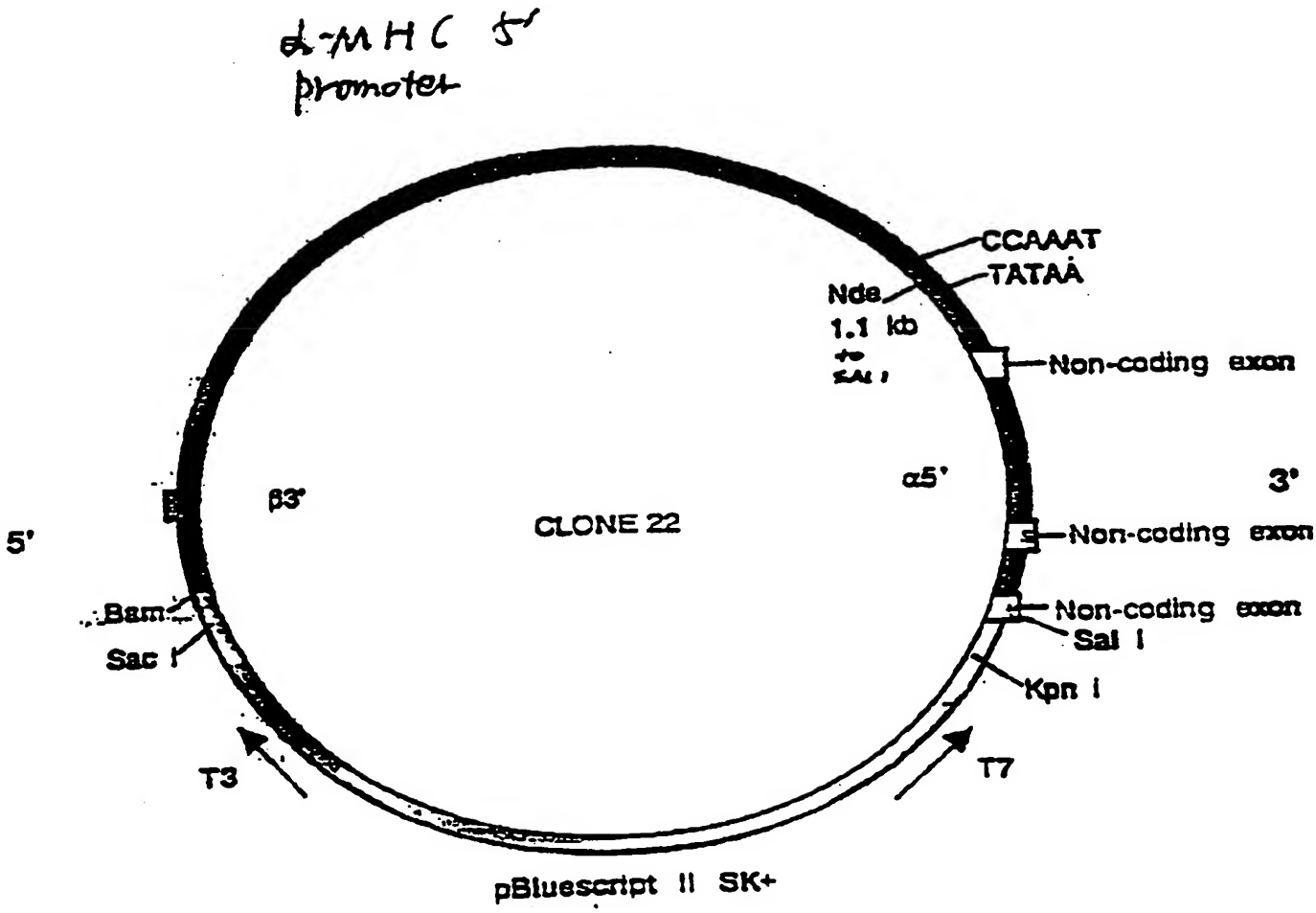
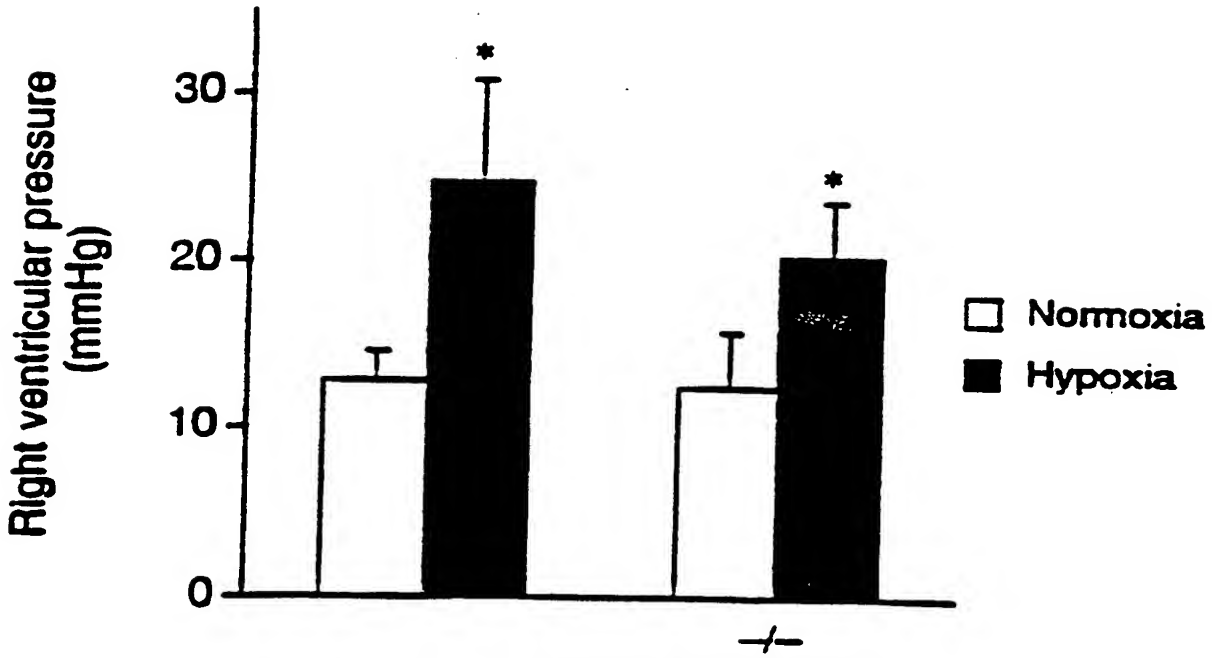

α-MHC 5'
promoter
CCAAAT
TATAA
Nde
1.1 kb
Non-coding exon
α5'
3'
β3'
CLONE 22
5'
Non-coding exon
Non-coding exon
Bam
Sac I
Sal I
Kpn I
T3
T7
pBluescript II SK+

FIG. 7


Right ventricular pressure (mmHg)
30
20
10
0
*
*
-/-
Normoxia
Hypoxia

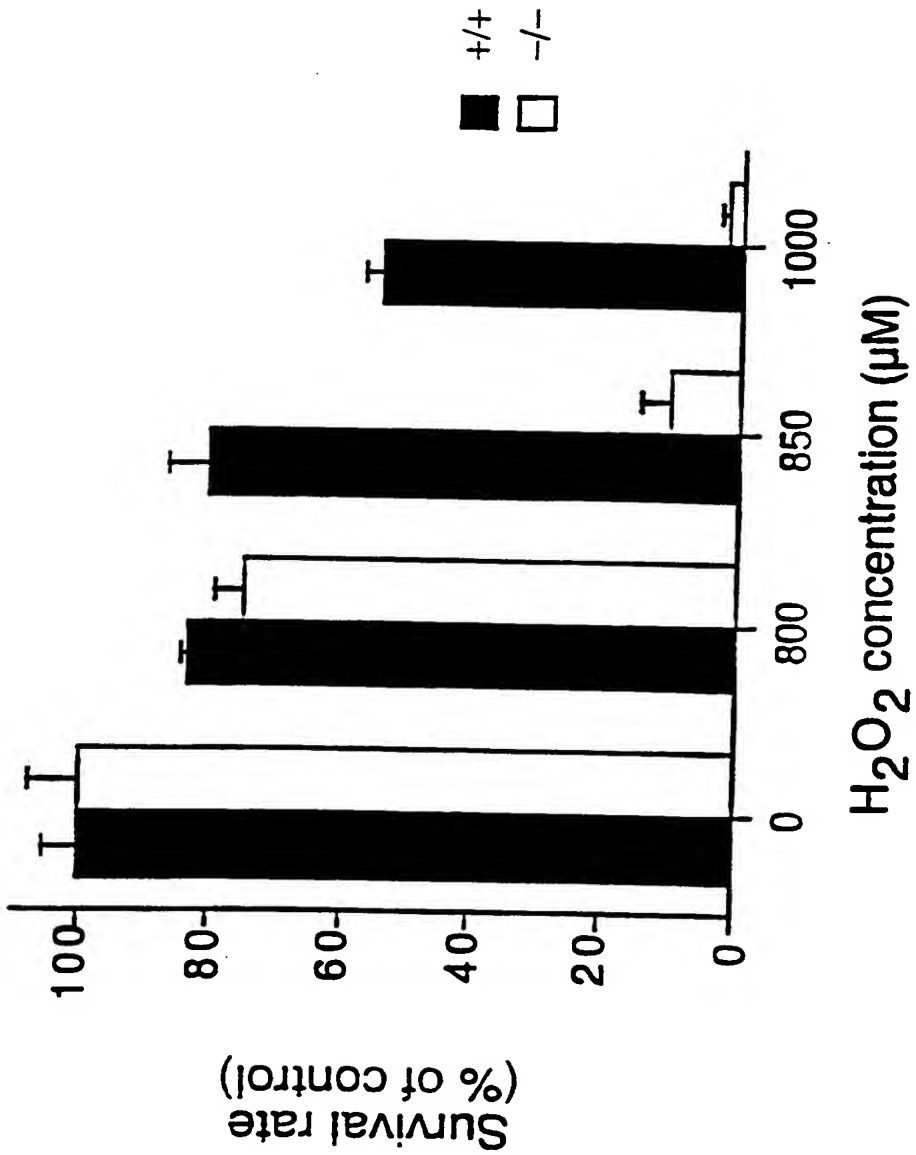

Survival rate
(% of control)
100
80
60
40
20
0
0
800
850
1000
H_2O_2 concentration (μM)
+/+
-/-

FIG. 8

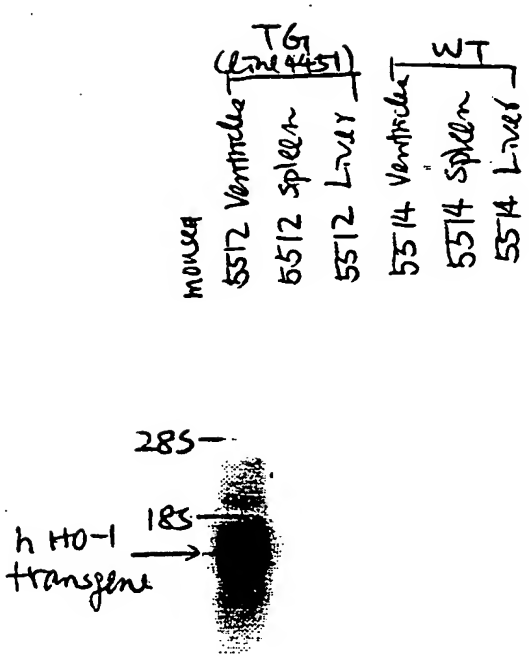
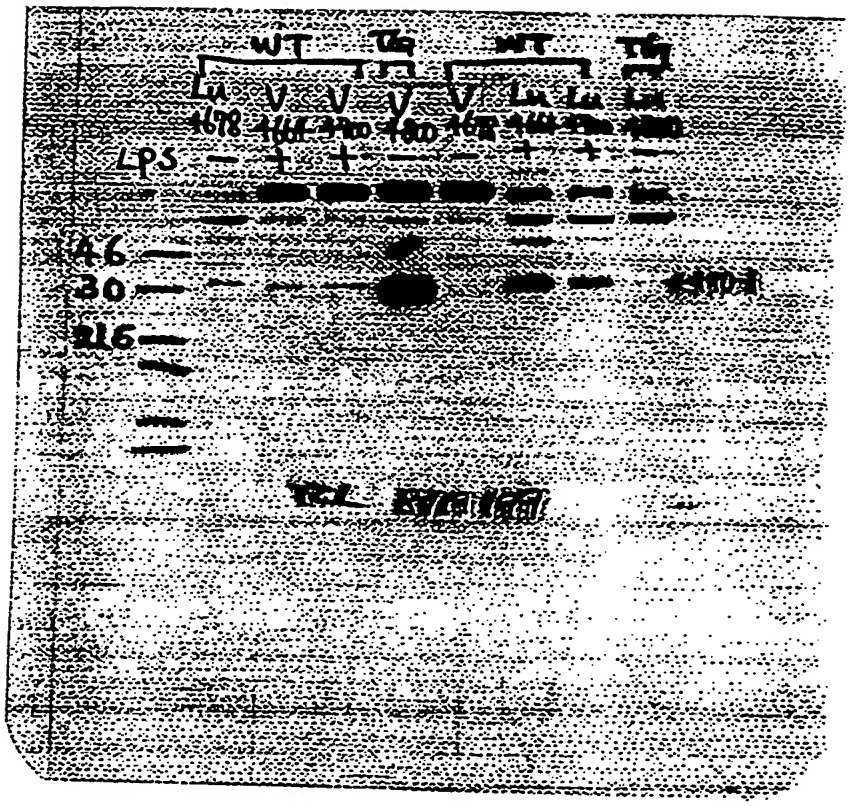

mouse
5512 Ventricle
5512 Spleen
5512 Liver
5514 Ventricle
5514 Spleen
5514 Liver
TG (Line #451)
WT
285
185
h HO-1 transgene

FIG. 10



V: Ventricles
Lu: Lung

SEQUENCE LISTING

<110> The President and Fellows of Harvard College

<120> INHIBITING CARDIOMYOCYTE DEATH

<130> 00246/235WO2

<150> US 60/121,946
<151> 1999-02-25

<150> US 60/098,377
<151> 1998-08-28

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<170> FastSEQ for Windows Version 3.0

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<213> Homo sapiens

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acgagcccag caccggccgg atg gag cgt ccg caa ccc gac agc atg ccc cag    113
                        Met Glu Arg Pro Gln Pro Asp Ser Met Pro Gln
                          1               5                  10

gat ttg tca gag gcc ctg aag gag gcc acc aag gag gtg cac acc cag      161
Asp Leu Ser Glu Ala Leu Lys Glu Ala Thr Lys Glu Val His Thr Gln
             15                  20                  25

gca gag aat gct gag ttc atg agg aac ttt cag aag ggc cag gtg acc      209
Ala Glu Asn Ala Glu Phe Met Arg Asn Phe Gln Lys Gly Gln Val Thr
         30                  35                  40

cga gac ggc ttc aag ctg gtg atg gcc tcc ctg tac cac atc tat gtg      257
Arg Asp Gly Phe Lys Leu Val Met Ala Ser Leu Tyr His Ile Tyr Val
     45                  50                  55

gcc ctg gag gag gag att gag cgc aac aag gag agc cca gtc ttc gcc      305
Ala Leu Glu Glu Glu Ile Glu Arg Asn Lys Glu Ser Pro Val Phe Ala
 60                  65                  70                  75

cct gtc tac ttc cca gaa gag ctg cac cgc aag gct gcc ctg gag cag      353
Pro Val Tyr Phe Pro Glu Glu Leu His Arg Lys Ala Ala Leu Glu Gln
                 80                  85                  90

gac ctg gcc ttc tgg tac ggg ccc cgc tgg cag gag gtc atc ccc tac      401
Asp Leu Ala Phe Trp Tyr Gly Pro Arg Trp Gln Glu Val Ile Pro Tyr
             95                  100                 105

aca cca gcc atg cag cgc tat gtg aag cgg ctc cac gag gtg ggg cgc      449
Thr Pro Ala Met Gln Arg Tyr Val Lys Arg Leu His Glu Val Gly Arg
         110                 115                 120

aca gag ccc gag ctg ctg gtg gcc cac gcc tac acc cgc tac ctg ggt      497
Thr Glu Pro Glu Leu Leu Val Ala His Ala Tyr Thr Arg Tyr Leu Gly
     125                 130                 135
```

```
gac ctg tct ggg ggc cag gtg ctc aaa aag att gcc cag aaa gcc ctg      545
Asp Leu Ser Gly Gly Gln Val Leu Lys Lys Ile Ala Gln Lys Ala Leu
140                 145                 150                 155

gac ctg ccc agc tct ggc gag ggc ctg gcc ttc ttc acc ttc ccc aac      593
Asp Leu Pro Ser Ser Gly Glu Gly Leu Ala Phe Phe Thr Phe Pro Asn
                160                 165                 170

att gcc agt gcc acc aag ttc aag cag ctc tac cgc tcc cgc atg aac      641
Ile Ala Ser Ala Thr Lys Phe Lys Gln Leu Tyr Arg Ser Arg Met Asn
            175                 180                 185

tcc ctg gag atg act ccc gca gtc agg cag agg gtg ata gaa gag gcc      689
Ser Leu Glu Met Thr Pro Ala Val Arg Gln Arg Val Ile Glu Glu Ala
        190                 195                 200

aag act gcg ttc ctg ctc aac atc cag ctc ttt gag gag ttg cag gag      737
Lys Thr Ala Phe Leu Leu Asn Ile Gln Leu Phe Glu Glu Leu Gln Glu
    205                 210                 215

ctg ctg acc cat gac acc aag gac cag agc ccc tca cgg gca cca ggg      785
Leu Leu Thr His Asp Thr Lys Asp Gln Ser Pro Ser Arg Ala Pro Gly
220                 225                 230                 235

ctt cgc cag cgg gcc agc aac aaa gtg caa gat tct gcc ccc gtg gag      833
Leu Arg Gln Arg Ala Ser Asn Lys Val Gln Asp Ser Ala Pro Val Glu
                240                 245                 250

act ccc aga ggg aag ccc cca ctc aac acc cgc tcc cag gct ccg ctt      881
Thr Pro Arg Gly Lys Pro Pro Leu Asn Thr Arg Ser Gln Ala Pro Leu
            255                 260                 265

ctc cga tgg gtc ctt aca ctc agc ttt ctg gtg gcg aca gtt gct gta      929
Leu Arg Trp Val Leu Thr Leu Ser Phe Leu Val Ala Thr Val Ala Val
        270                 275                 280

ggg ctt tat gcc atg tgaatgcagg catgctggct cccagggcca tgaactttgt      984
Gly Leu Tyr Ala Met
    285

ccggtggaag gccttctttc tagagaggga attctcttgg ctggcttcct taccgtgggc   1044
actgaaggct ttcagggcct ccagccctct cactgtgtcc ctctctctgg aaaggaggaa   1104
ggagcctatg gcatcttccc caacgaaaag cacatccagg caatggccta aacttcagag   1164
ggggcgaagg ggtcagccct gcccttcagc atcctcagtt cctgcagcag agcctggaag   1224
acaccctaat gtggcagctg tctcaaacct ccaaaagccc tgagtttcaa gtatccttgt   1284
tgacacggcc atgaccactt tccccgtggg ccatggcaat ttttacacaa acctgaaaag   1344
atgttgtgtc ttgtgttttt gtcttatttt tgttggagcc actctgttcc tggctcagcc   1404
tcaaatgcag tatttttgtt gtgttctgtt gttttttatag cagggttggg gtggtttttg   1464
agccatgcgt gggtggggag ggaggtgttt aacggcactg tggccttggt ctaacttttg   1524
tgtgaaataa taaacaacat tgtctg                                       1550
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<212> PRT
<213> Homo sapiens

<400> 2

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Leu Lys Glu Ala Thr Lys Glu Val His Thr Gln Ala Glu Asn Ala Glu
            20                  25                  30
Phe Met Arg Asn Phe Gln Lys Gly Gln Val Thr Arg Asp Gly Phe Lys
        35                  40                  45
Leu Val Met Ala Ser Leu Tyr His Ile Tyr Val Ala Leu Glu Glu Glu
    50                  55                  60
Ile Glu Arg Asn Lys Glu Ser Pro Val Phe Ala Pro Val Tyr Phe Pro
65                  70                  75                  80
```

```
Glu Glu Leu His Arg Lys Ala Ala Leu Glu Gln Asp Leu Ala Phe Trp
                85                  90                  95
Tyr Gly Pro Arg Trp Gln Glu Val Ile Pro Tyr Thr Pro Ala Met Gln
            100                 105                 110
Arg Tyr Val Lys Arg Leu His Glu Val Gly Arg Thr Glu Pro Glu Leu
        115                 120                 125
Leu Val Ala His Ala Tyr Thr Arg Tyr Leu Gly Asp Leu Ser Gly Gly
    130                 135                 140
Gln Val Leu Lys Lys Ile Ala Gln Lys Ala Leu Asp Leu Pro Ser Ser
145                 150                 155                 160
Gly Glu Gly Leu Ala Phe Phe Thr Phe Pro Asn Ile Ala Ser Ala Thr
                165                 170                 175
Lys Phe Lys Gln Leu Tyr Arg Ser Arg Met Asn Ser Leu Glu Met Thr
            180                 185                 190
Pro Ala Val Arg Gln Arg Val Ile Glu Glu Ala Lys Thr Ala Phe Leu
        195                 200                 205
Leu Asn Ile Gln Leu Phe Glu Glu Leu Gln Glu Leu Leu Thr His Asp
    210                 215                 220
Thr Lys Asp Gln Ser Pro Ser Arg Ala Pro Gly Leu Arg Gln Arg Ala
225                 230                 235                 240
Ser Asn Lys Val Gln Asp Ser Ala Pro Val Glu Thr Pro Arg Gly Lys
                245                 250                 255
Pro Pro Leu Asn Thr Arg Ser Gln Ala Pro Leu Leu Arg Trp Val Leu
            260                 265                 270
Thr Leu Ser Phe Leu Val Ala Thr Val Ala Val Gly Leu Tyr Ala Met
        275                 280                 285
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<222> (85)...(1023)

<400> 3

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gagcaagaac cacacccagc agca atg tca gcg gaa gtg gaa acc tca gag         111
                           Met Ser Ala Glu Val Glu Thr Ser Glu
                             1               5

ggg gta gac gag tca gaa aaa aag aac tct ggg gcc cta gaa aag gag       159
Gly Val Asp Glu Ser Glu Lys Lys Asn Ser Gly Ala Leu Glu Lys Glu
 10                  15                  20                  25

aac caa atg aga atg gct gac ctc tca gag ctc ctg aag gaa ggg acc       207
Asn Gln Met Arg Met Ala Asp Leu Ser Glu Leu Leu Lys Glu Gly Thr
                 30                  35                  40

aag gaa gca cac gac cgg gca gaa aac acc cag ttt gtc aag gac ttc       255
Lys Glu Ala His Asp Arg Ala Glu Asn Thr Gln Phe Val Lys Asp Phe
             45                  50                  55

ttg aaa ggc aac att aag aag gag ctg ttt aag ctg gcc acc acg gca       303
Leu Lys Gly Asn Ile Lys Lys Glu Leu Phe Lys Leu Ala Thr Thr Ala
         60                  65                  70

ctt tac ttc aca tac tca gcc ctc gag gag gaa atg gag cgc aac aag       351
Leu Tyr Phe Thr Tyr Ser Ala Leu Glu Glu Glu Met Glu Arg Asn Lys
     75                  80                  85

gac cat cca gcc ttt gcc cct ttg tac ttc ccc atg gag ctg cac cgg       399
Asp His Pro Ala Phe Ala Pro Leu Tyr Phe Pro Met Glu Leu His Arg
 90                  95                 100                 105
```

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aag gag gcg ctg acc aag gac atg gag tat ttc ttt ggt gaa aac tgg      447
Lys Glu Ala Leu Thr Lys Asp Met Glu Tyr Phe Phe Gly Glu Asn Trp
                110                 115                 120

gag gag cag gtg cag tgc ccc aag gct gcc cag aag tac gtg gag cgg      495
Glu Glu Gln Val Gln Cys Pro Lys Ala Ala Gln Lys Tyr Val Glu Arg
            125                 130                 135

atc cac tac ata ggg cag aac gag ccg gag cta ctg gtg gcc cat gca      543
Ile His Tyr Ile Gly Gln Asn Glu Pro Glu Leu Leu Val Ala His Ala
        140                 145                 150

tac acc cgc tac atg ggg gat ctc tcg ggg ggc cag gtg ctg aag aag      591
Tyr Thr Arg Tyr Met Gly Asp Leu Ser Gly Gly Gln Val Leu Lys Lys
    155                 160                 165

gtg gcc cag cga gca ctg aaa ctc ccc agc aca ggg gaa ggg acc cag      639
Val Ala Gln Arg Ala Leu Lys Leu Pro Ser Thr Gly Glu Gly Thr Gln
170                 175                 180                 185

ttc tac ctg ttt gag aat gtg gac aat gcc cag cag ttc aag cag ctc      687
Phe Tyr Leu Phe Glu Asn Val Asp Asn Ala Gln Gln Phe Lys Gln Leu
                190                 195                 200

tac cgg gcc agg atg aac gcc ctg gac ctg aac atg aag acc aaa gag      735
Tyr Arg Ala Arg Met Asn Ala Leu Asp Leu Asn Met Lys Thr Lys Glu
            205                 210                 215

agg atc gtg gag gcc aac aag gct ttt gag tat aac atg cag ata ttc      783
Arg Ile Val Glu Ala Asn Lys Ala Phe Glu Tyr Asn Met Gln Ile Phe
        220                 225                 230

aat gaa ctg gac cag gcc ggc tcc aca ctg gcc aga gag acc ttg gag      831
Asn Glu Leu Asp Gln Ala Gly Ser Thr Leu Ala Arg Glu Thr Leu Glu
    235                 240                 245

gat ggg ttc cct gta cac gat ggg aaa gga gac atg cgt aaa tgc cct      879
Asp Gly Phe Pro Val His Asp Gly Lys Gly Asp Met Arg Lys Cys Pro
250                 255                 260                 265

ttc tac gct gct gaa caa gac aaa ggg ctg gag ggc agc ctg tcc ctt      927
Phe Tyr Ala Ala Glu Gln Asp Lys Gly Leu Glu Gly Ser Leu Ser Leu
                270                 275                 280

ccg aca agc tat gct gtg ctg agg aag ccc agc ctc cag ttc atc ctg      975
Pro Thr Ser Tyr Ala Val Leu Arg Lys Pro Ser Leu Gln Phe Ile Leu
            285                 290                 295

gcc gct ggt gtg gcc cta gct gct gga ctc ttg gcc tgg tac tac atg     1023
Ala Ala Gly Val Ala Leu Ala Ala Gly Leu Leu Ala Trp Tyr Tyr Met
        300                 305                 310

tgaagcaccc atcatgccac accggtaccc tcctcccgac tgaccactgg cctacccctt   1083
tctccagccc tgactaaact accacctcag gtgacttttt aaaaaatgct gggtttaaga   1143
aaggcaacca ataaaagaga tgctagagcc tcgtctgaca gcatcctctc tatgggccat   1203
attccgcact gggcacaggc cgtcaccctg ggagcagtcg gcacagtgca gcaagcctgg   1263
cccccgaccc agctctactc caggcttcca cacttctggg ccctaggctg cttccggtag   1323
tccctgtttt tgcagtacat gggtgactat ctcccctgtt ggaggtgagt ggcctgtaag   1383
tccaagctgt gcgaggggcc cttgctggat gctgctgtac aacttctggg cctctcttgg   1443
accctgggag tgagggtggg tgtgggtgga agcctcagag gccttgggag ctcatccctc   1503
tcacccagaa tccctctaac ccttgggtgc ggtttgctca gccccagctt atctcctcct   1563
ccgcctgtgt aaatgctcca gcactcaata aagtgggctt tgcaagctaa aaaaaaaaaa   1623
aaaa                                                               1627
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<210> 4
<211> 313
<212> PRT

<213> Homo sapiens

<400> 4

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            20                  25                  30
Leu Ser Glu Leu Leu Lys Glu Gly Thr Lys Glu Ala His Asp Arg Ala
        35                  40                  45
Glu Asn Thr Gln Phe Val Lys Asp Phe Leu Lys Gly Asn Ile Lys Lys
    50                  55                  60
Glu Leu Phe Lys Leu Ala Thr Thr Ala Leu Tyr Phe Thr Tyr Ser Ala
65                  70                  75                  80
Leu Glu Glu Glu Met Glu Arg Asn Lys Asp His Pro Ala Phe Ala Pro
                85                  90                  95
Leu Tyr Phe Pro Met Glu Leu His Arg Lys Glu Ala Leu Thr Lys Asp
            100                 105                 110
Met Glu Tyr Phe Phe Gly Glu Asn Trp Glu Glu Gln Val Gln Cys Pro
        115                 120                 125
Lys Ala Ala Gln Lys Tyr Val Glu Arg Ile His Tyr Ile Gly Gln Asn
    130                 135                 140
Glu Pro Glu Leu Leu Val Ala His Ala Tyr Thr Arg Tyr Met Gly Asp
145                 150                 155                 160
Leu Ser Gly Gly Gln Val Leu Lys Lys Val Ala Gln Arg Ala Leu Lys
                165                 170                 175
Leu Pro Ser Thr Gly Glu Gly Thr Gln Phe Tyr Leu Phe Glu Asn Val
            180                 185                 190
Asp Asn Ala Gln Gln Phe Lys Gln Leu Tyr Arg Ala Arg Met Asn Ala
        195                 200                 205
Leu Asp Leu Asn Met Lys Thr Lys Glu Arg Ile Val Glu Ala Asn Lys
    210                 215                 220
Ala Phe Glu Tyr Asn Met Gln Ile Phe Asn Glu Leu Asp Gln Ala Gly
225                 230                 235                 240
Ser Thr Leu Ala Arg Glu Thr Leu Glu Asp Gly Phe Pro Val His Asp
                245                 250                 255
Gly Lys Gly Asp Met Arg Lys Cys Pro Phe Tyr Ala Ala Glu Gln Asp
            260                 265                 270
Lys Gly Leu Glu Gly Ser Leu Ser Leu Pro Thr Ser Tyr Ala Val Leu
        275                 280                 285
Arg Lys Pro Ser Leu Gln Phe Ile Leu Ala Ala Gly Val Ala Leu Ala
    290                 295                 300
Ala Gly Leu Leu Ala Trp Tyr Tyr Met
305                 310
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<211> 2225
<212> DNA
<213> Rattus rattus

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<222> (1062)...(1931)

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cttttctggt gtgtttggat attccatgtt tgttttggtg ggagaattgg gctccgatga    180
tggcatgtag tcttggtttc tgttgcttgg tttcctgcgc ttgcctctcg ccatcagatt    240
atctctagtg ttactttgtt ctgctatttc tgacagtggc tagactgtcc tataagcctg    300
tgtgtcagga gtgctgtaga ccttttttcc tctctttcag tcagttatgg gacagagtgt    360
tctgcttttg ggcgtgtagt ttttcctctc tacaggtctt cagctgttcc tgtgggcctg    420
tgtcttgagt tcaccaggca gctttcttgc agcagaaaat ttggtcatac ctgtgatcct    480
gaggctcaag ttcgctcgtg gggtgctgtc caggggctct ctgcagcggg cacaaccagg    540
aagacctgtg cggccccttc cggagcttca gtgcaccagg gttccagatg gcctttggcg    600
ttttcctctg gcgtccgaga tgtatgtaca gagagcagtc tcttctggtt tcccaggctt    660
gtctgcctct ctgaaggttc agctctccct cccacgggat ttgggtgcag agaactgttt    720
atccggtctg tttctttcag gttccggtgg tgtctcaggc aggtgtcgtt cctgcgccct    780
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gggtctctct ctgagaattt catttttaaa tcattcatta aaatgtcatg acttgatgtc    960
ctgctgtccg tctcacgccc tcagctgtaa cagtgccgag ggagtcactg aagaagagac   1020
tgaatgacca gagtatgggc agcacagaca actcaacaaa a atg tct tca gag gtg   1076
                                              Met Ser Ser Glu Val
                                                1               5

gag act gcg gag gcc gta gat gag tca gag aag aac tct atg gca tca     1124
Glu Thr Ala Glu Ala Val Asp Glu Ser Glu Lys Asn Ser Met Ala Ser
                  10                  15                  20

gag aag gaa aac cat tcc aaa ata gca gac ttt tct gat ctt ctg aag     1172
Glu Lys Glu Asn His Ser Lys Ile Ala Asp Phe Ser Asp Leu Leu Lys
              25                  30                  35

gaa ggg aca aag gaa gca gat gac cgg gca gaa aat acc cag ttt gtc     1220
Glu Gly Thr Lys Glu Ala Asp Asp Arg Ala Glu Asn Thr Gln Phe Val
          40                  45                  50

aaa gac ttc ttg aaa gga aac att aag aag gag cta ttt aag ctg gcc     1268
Lys Asp Phe Leu Lys Gly Asn Ile Lys Lys Glu Leu Phe Lys Leu Ala
      55                  60                  65

acc act gca ctt tca tac tca gcc cct gag gag gaa atg gat tca ctg     1316
Thr Thr Ala Leu Ser Tyr Ser Ala Pro Glu Glu Glu Met Asp Ser Leu
  70                  75                  80                  85

acc aag gac atg gag tac ttc ttt ggt gaa aac tgg gag gaa aaa gtg     1364
Thr Lys Asp Met Glu Tyr Phe Phe Gly Glu Asn Trp Glu Glu Lys Val
                  90                  95                 100

aag tgc tct gaa gct gcc cag acg tat gtg gat cag att cac tat gta     1412
Lys Cys Ser Glu Ala Ala Gln Thr Tyr Val Asp Gln Ile His Tyr Val
              105                 110                 115

ggg caa aat gag cca gag cat ctg gtg gcc cat act tac tct act tac     1460
Gly Gln Asn Glu Pro Glu His Leu Val Ala His Thr Tyr Ser Thr Tyr
          120                 125                 130

atg ggg gga aac ctt tca ggg gac cag gta ctg aag aag gag acc cag     1508
Met Gly Gly Asn Leu Ser Gly Asp Gln Val Leu Lys Lys Glu Thr Gln
      135                 140                 145

ccg gtc ccc ttc act agg gaa ggg act cag ttc tac ctg ttt gag cat     1556
Pro Val Pro Phe Thr Arg Glu Gly Thr Gln Phe Tyr Leu Phe Glu His
150                 155                 160                 165

gta gac aat gct aag caa ttc aag cta ttc tac tgc gct aga ttg aat     1604
Val Asp Asn Ala Lys Gln Phe Lys Leu Phe Tyr Cys Ala Arg Leu Asn
                  170                 175                 180

gcc ttg gac ctg aat ttg aag acc aaa gag agg att gtg gag gaa gcc     1652
Ala Leu Asp Leu Asn Leu Lys Thr Lys Glu Arg Ile Val Glu Glu Ala
              185                 190                 195

acc aaa gcc ttt gaa tat aat atg cag ata ttc agt gaa ctg gac cag     1700
Thr Lys Ala Phe Glu Tyr Asn Met Gln Ile Phe Ser Glu Leu Asp Gln
          200                 205                 210

gca ggc tcc ata cca gta aga gaa acc cta aag aat ggg ctc tca ata     1748
Ala Gly Ser Ile Pro Val Arg Glu Thr Leu Lys Asn Gly Leu Ser Ile
      215                 220                 225

ctt gat ggg aag gga ggt gta tgc aaa tgt ccc ttt aat gct gct cag     1796
Leu Asp Gly Lys Gly Gly Val Cys Lys Cys Pro Phe Asn Ala Ala Gln
230                 235                 240                 245
```

```
cca gac aaa ggt acc ctg gga ggc agc aac tgc cct ttc cag atg tcc     1844
Pro Asp Lys Gly Thr Leu Gly Gly Ser Asn Cys Pro Phe Gln Met Ser
                250                 255                 260

atg gcc ttg ctg agg aag cct aac ttg cag ctc att cta gtt gcc agt     1892
Met Ala Leu Leu Arg Lys Pro Asn Leu Gln Leu Ile Leu Val Ala Ser
            265                 270                 275

atg gcc ttg gta gct gga ctt tta gcc tgg tac tac atg tgaagggcct      1941
Met Ala Leu Val Ala Gly Leu Leu Ala Trp Tyr Tyr Met
        280                 285                 290

gtcaagttgt ttgcatccta tctcaacatc ctaccacttg ttccttcccc acctccacct   2001
ctgcctagaa ctaccacctc aggtgacatt tttaatgttg ggtttgagaa aatgagcaac   2061
caataaaaga cagaccctag aaaaaagtca tgacttaagt ggcacgggga cacctaaagt   2121
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cctactacta ttaataataa atgctacaca atgcataata aaaa                    2225
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<210> 6
<211> 290
<212> PRT
<213> Rattus rattus

<400> 6

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            20                  25                  30
Ser Asp Leu Leu Lys Glu Gly Thr Lys Glu Ala Asp Asp Arg Ala Glu
        35                  40                  45
Asn Thr Gln Phe Val Lys Asp Phe Leu Lys Gly Asn Ile Lys Lys Glu
    50                  55                  60
Leu Phe Lys Leu Ala Thr Thr Ala Leu Ser Tyr Ser Ala Pro Glu Glu
65                  70                  75                  80
Glu Met Asp Ser Leu Thr Lys Asp Met Glu Tyr Phe Phe Gly Glu Asn
                85                  90                  95
Trp Glu Glu Lys Val Lys Cys Ser Glu Ala Ala Gln Thr Tyr Val Asp
            100                 105                 110
Gln Ile His Tyr Val Gly Gln Asn Glu Pro Glu His Leu Val Ala His
        115                 120                 125
Thr Tyr Ser Thr Tyr Met Gly Gly Asn Leu Ser Gly Asp Gln Val Leu
    130                 135                 140
Lys Lys Glu Thr Gln Pro Val Pro Phe Thr Arg Glu Gly Thr Gln Phe
145                 150                 155                 160
Tyr Leu Phe Glu His Val Asp Asn Ala Lys Gln Phe Lys Leu Phe Tyr
                165                 170                 175
Cys Ala Arg Leu Asn Ala Leu Asp Leu Asn Leu Lys Thr Lys Glu Arg
            180                 185                 190
Ile Val Glu Glu Ala Thr Lys Ala Phe Glu Tyr Asn Met Gln Ile Phe
        195                 200                 205
Ser Glu Leu Asp Gln Ala Gly Ser Ile Pro Val Arg Glu Thr Leu Lys
    210                 215                 220
Asn Gly Leu Ser Ile Leu Asp Gly Lys Gly Gly Val Cys Lys Cys Pro
225                 230                 235                 240
Phe Asn Ala Ala Gln Pro Asp Lys Gly Thr Leu Gly Gly Ser Asn Cys
                245                 250                 255
Pro Phe Gln Met Ser Met Ala Leu Leu Arg Lys Pro Asn Leu Gln Leu
            260                 265                 270
Ile Leu Val Ala Ser Met Ala Leu Val Ala Gly Leu Leu Ala Trp Tyr
        275                 280                 285
Tyr Met
    290
```

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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(74) Agent: BEATTIE, Ingrid, A.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: AU, CA, IL, JP, NO, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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(54) Title: INHIBITING CARDIOMYOCYTE DEATH

(57) Abstract

The invention features methods of inhibiting cardiomyocyte death in a mammal by administering to the myocardium of the mammal a heme oxygenase (HO). Methods of preserving an organ for transplantation and methods of inhibiting vascular stenosis are also within the invention.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/19823

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/44 A61K48/00 A61P9/10 //A61K38/18,A61K31/555

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABRAHAM, N. G. (1) ET AL: "Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: Protective effect against heme and hemoglobin toxicity." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1995) VOL. 92, NO. 15, PP. 6798-6802. , XP002100374 the whole document ---	1-12
X	WO 98 08566 A (WISCONSIN MED COLLEGE INC ;UNIV DUKE (US)) 5 March 1998 (1998-03-05) page 4, line 23 -page 6, line 12 page 7, line 24 -page 8, line 2 page 9, line 19 -page 10, line 18 page 55, line 2 - line 30 page 57, line 22 - line 28 --- -/--	14-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2000	09/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stein, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 36615 A (HARVARD COLLEGE) 9 October 1997 (1997-10-09) page 2, line 11 - line 36 page 7, line 16 -page 8, line 13 page 16, line 18 -page 17, line 36 page 21, line 14 -page 22, line 8 claims 1-6 ---	14-23
A	MAULIK N ET AL: "Nitric oxide/carbon monoxide. A molecular switch for myocardial preservation during ischemia." CIRCULATION, (1996 NOV 1) 94 (9 SUPPL) II398-406. , XP000876907 the whole document ---	1-13
A	ABRAHAM, NADER G. (1): "Manipulation of heme oxygenase expression by gene transfer and metals: Implications in cell injury and repair." JOURNAL OF NEUROCHEMISTRY, (1998) VOL. 70, NO. SUPPL. 1, PP. S45. MEETING INFO.: 29TH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR NEUROCHEMISTRY DENVER, COLORADO, USA MARCH 7-11, 1998 AMERICAN SOCIETY FOR NEUROCHEMISTRY. , XP000876935 the whole document ---	1-13
A	LONG, XILIN ET AL: "Hypoxia-induced expression of heme oxygenase gene expression in cultured neonatal rat cardiac myocytes." CIRCULATION, (1995) VOL. 92, NO. 8 SUPPL., PP. I653-I654, XP000876926 the whole document ---	1-13
A	HOSHIDA, SHIRO ET AL: "Heme oxygenase -1 as a culture shock protein in rat neonatal cardiomyocytes." JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, (1994) VOL. 26, NO. 11, PP. CCXII, XP000876927 the whole document ---	1-13
A	BORGER DR: "Induction of HSP 32 gene in hypoxic cardiomyocytes is attenuated by treatment with N-acetyl-L-cysteine" AMERICAN JOURNAL OF PHYSIOLOGY, vol. 274, no. 3 Pt 2, March 1998 (1998-03), pages H965-73, XP002131421 the whole document --- -/--	1-14

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MORITA T ET AL: "Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells" JOURNAL OF BIOLOGICAL CHEMISTRY, (26 DEC 1997) VOL. 272, NO. 52, PP. 32804-32809, XP002131422 the whole document ---	24,25
P,X	SOARES M P ET AL: "Expression of heme oxygenase -1 can determine cardiac xenograft survival." NATURE MEDICINE, (1998 SEP) 4 (9) 1073-7., XP002131423 the whole document -----	13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 19823

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-8, 14-25 are directed to a method of treatment of the human/animal the search has been carried out and based based on the alleged effects of the compound /composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No
PCT/US 99/19823

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9808566 A	05-03-1998	AU 4054297 A	19-03-1998
		EP 0963219 A	15-12-1999
WO 9736615 A	09-10-1997	US 5888982 A	30-03-1999

CORRECTED VERSION*

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A3

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*(Referred to in PCT Gazette No. 32/2000, Section II)

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INHIBITING CARDIOMYOCYTE DEATH

Related Application Information

This application claims priority from provisional application no. 60/121,946, filed on February 25, 1999, and provisional application no. 60/098,377, filed on August 28, 1998.

Statement as to Federally Sponsored Research

This invention was made with U.S. Government support under National Institutes of Health grants RO1 GM53249, KO8 HL03274, and KO8 HL03194. The government has certain rights in the invention.

Background of the Invention

The invention relates to treatment of cardiovascular disease.

Myocardial infarction is one of the most common diagnoses of hospitalized patients in western countries. In the United States, over 1.5 million myocardial infarctions occur annually, and mortality from acute myocardial infarction is approximately 25 per cent. Thrombolytic therapy and reperfusion of ischemic myocardium, e.g., using percutaneous transluminal coronary angioplasty (PTCA), have decreased mortality rates among patients who have suffered an acute myocardial infarction. Nevertheless, myocardial damage and heart failure resulting from a failure to achieve early revascularization or from reperfusion injury remain a significant clinical problem.

Summary of the Invention

The invention features methods of minimizing myocardial damage by salvaging hypoxic myocardial tissue before it becomes irreversibly injured. For example, a method of inhibiting cardiomyocyte death in a mammal, e.g., a human, who has suffered a myocardial infarction or who has myocarditis is carried out by locally

administering to the myocardium of the mammal a heme oxygenase (HO) polypeptide. Preferably, the HO polypeptide has the amino acid sequence of a naturally-occurring heme oxygenase-1 (HO-1), heme oxygenase-2 (HO-2), or heme oxygenase-3 (HO-3), or a biologically active fragment thereof.

Compositions, such as hemin, hemoglobin, or heavy metals, e.g., tin or nickel, that increase production of endogenous HO, are also administered to inhibit cardiomyocyte death or damage. For example, overexpression of HO-1 is induced in vascular cells by exposure to heme, heavy metals, endotoxin and hyperoxia, hyperthermia, shear stress and strain, UV light, or reactive oxygen species. By "overexpression" is meant a level of protein production that at least 20% greater than that present in the tissue under normal physiologic conditions. Preferably, the level of HO-1 expression in vascular tissue in the presence of an inducing agent is at least 20% greater than that in the absence of the inducing agent; more preferably, the level of expression is at least 50% greater, more preferably, the level of expression is at least 100% greater, and most preferably, the level of expression is at least 200% greater than that in the absence of an inducing agent. Inhibition of cardiomyocyte death is also achieved by locally administering to the myocardium of a mammal a DNA encoding a HO. HO expression by target cell, e.g., VSMC, is increased by administering to the cells exogenous DNA encoding HO, e.g., a plasmid containing DNA encoding human HO-1 or HO-2 under the control of a strong constitutive promoter. Oxidative stress leads to cell death by apoptosis and/or necrosis. By neutralizing reactive oxygen species, HO reduces cardiomyocyte damage and death due to oxidative stress.

The invention also includes a method of inhibiting cardiomyocyte death *in vitro* by contacting cardiomyocytes with an HO or DNA encoding an HO. For example, a method of preserving isolated myocardial tissue, e.g., a donor heart to be used for transplantation, is carried out by bathing or perfusing the tissue with a solution containing an HO or a DNA encoding an HO. The method allows prolonged storage of organs after removal from the donor and prior to transplantation into a recipient by reducing irreversible ischemic tissue damage. By "isolated myocardial tissue" is meant tissue that has been removed from a living or recently deceased mammal. Preferably, a donor heart is preserved in an HO solution for 0.5-6 hours prior to transplantation. More preferably, the organ is preserved for greater than 6 hours, e.g., 8, 10, 12, and up to 24 hours.

A method of inhibiting vascular stenosis or restenosis in a mammal, e.g., a human, is also within the invention. The method is carried out by locally administering to the site of a vascular injury or a site which is at risk of developing a stenotic lesion a compound which inhibits expression of HO-1, and as a result, VSMC proliferation. To inhibit vascular stenosis or restenosis (which occurs at a relatively late stage after the occurrence of a vascular injury), the compound is administered at least one month after an injury such as surgery or angioplasty. For example, such treatment is administered 3 weeks to several months (e.g., 2 months or 3 months) post-injury. Preferably, the compound inhibits transcription of the gene encoding HO-1 or inhibits translation of HO-1 mRNA into an HO-1 polypeptide in a vascular cell, e.g., a vascular smooth muscle cell (VSMC), of the mammal. The vascular cell is preferably an aortic smooth muscle cell, e.g., an aortic smooth muscle cell located in the region of an artery

affected by vascular stenosis or restenosis such as the site of balloon angioplasty or coronary bypass surgery. For example, transforming growth factor-β1 (TGF-β1) is administered to inhibit production of HO-1 mRNA and HO gene product.

For antisense therapy, the compound is a antisense nucleic acid molecule containing at least 10 nucleotides, the sequence of which is complementary to an mRNA encoding all or part of a wild type HO polypeptide. Preferably, the compound, e.g., an antisense oligonucleotide or antisense RNA produced from an antisense template, inhibits HO expression. The antisense nucleic acid inhibits HO expression by inhibiting translation of HO mRNA. For example, antisense therapy is carried out by administering a single stranded nucleic acid complementary at least a portion of HO mRNA to interfere with the translation of mRNA into protein, thus reducing the amount of functional HO produced in the cell. The method includes the step of identifying a mammal having undesired vascular stenosis or restenosis or at risk of developing such a condition. For example, the mammal to be treated is one who needs or has recently undergone PTCA, coronary artery bypass surgery, other vascular injury, that stimulates vascular smooth muscle cell proliferation that results in undesired vascular stenosis or restenosis.

For treatment of a vascular injury soon after the injury or stress has occurred, an HO polypeptide, e.g., HO-1, or a nucleic acid encoding an HO polypeptide, is administered to a mammal within minutes until approximately one week post-injury. Augmentation of the level of HO in injured vascular tissue shortly after the injury has occurred inhibits an initial increase in local VSMC proliferation post-injury. For example, such early

stage intervention is carried out within 24 hours post-injury.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

The drawings will first be briefly described.

Drawings

Fig. 1 is a diagram of the targeted gene disruption strategy used in making an HO-1-deficient mouse.

Fig. 2 is a bar graph showing that hypoxia increases hematocrit in HO-1 +/+ and -/- mice.

Fig. 3 is a bar graph showing that hypoxia markedly increases ventricular weight in HO-1 -/- mice.

Fig. 4 is a diagram of the mouse model of vein graft stenosis.

Fig. 5A is a line graph showing luminal occlusion of an artery into which a vein patch has been grafted.

Fig. 5B is a diagram of a vein graft.

Fig. 6 is a diagram of plasmid containing a myosin heavy chain promoter which directs cardiospecific expression of a polypeptide-encoding DNA to which it is operably linked.

Fig. 7 is a bar graph showing that chronic hypoxia increases right ventricular systolic pressure. Wild type (+/+) and HO-1-deficient (-/-) mice were exposed to normoxia or chronic hypoxia (10% oxygen). Right ventricular pressure was measured under normoxic conditions (open bars) or after five weeks of hypoxia (filled bars). Error bars indicate standard deviation. *$P<0.05$ vs. animals exposed to normoxia within the same group ($n = 5$ in each group).

Fig. 8 is a bar graph showing that HO-1 -/- arterial smooth muscle cells are more sensitive to

oxidative stress compared to wild type smooth muscle cells.

Fig. 9 is an autoradiograph of a Northern blot assay showing expression of a human HO-1 (hHO-1) transgene in a transgenic mouse.

Fig. 10 is an autoradiograph of a Western blot showing the presence of a hHO-1 gene product in tissues of HO-1 transgenic mice.

HO-1-deficient mice

HO-1-deficient (HO-1-/-) mice were produced using a standard targeted gene deletion strategy to delete exon 3 (Fig. 1). The murine HO-1 gene contains 5 exons and 4 introns, spanning approximately 7 kilobases (kb). The targeting construct was made by deleting the largest exon (exon 3) which contains 492 nucleotides out of the 867 nucleotides of the entire open reading frame. This deletion renders the HO-1 enzyme non-functional. An *Xho*I/*BamH*I fragment of the *neo* cassette from pMC1*neo* PolyA plasmid was subcloned into pBluescript II SK (Stratagene, La Jolla, CA) to generate pBS-*neo*. To generate pBS-*neo*-HO-1, the 3 kb *Xho*I fragment of the HO-1 gene spanning from exon 1 to the end of intron 2 was subcloned into the *Xho*I site of pBS-*neo* in the same orientation as the *neo* cassette. The 4 kb HO-1 *BamH*I-*EcoR*I fragment containing a small portion of intron 3, exon 4, and exon 5 was subcloned into *BamH*I and *EcoR*I site of pPGK-TK to generate pPGK-TK-HO-1. The 7 kb *BamH*I-*Cla*I fragment (filled in with Klenow) from pPGK-TK-HO-1 was then subcloned into *BamH*I and *Xba*I sites (filled in with Klenow) sites of pBS-*neo*-HO-1 to generate the HO-1 targeting construct. The linearized targeting construct was transfected into murine D3 embryonic stem (ES) cells, and a clone with the correct homologous recombination (yielding the appropriately disrupted HO-1 gene) injected into blastocysts and used to generate HO-1

deficient mice. The survival rate of HO-1 -/- mice was 25% of the expected survival rate, and the mice were grossly normal. The mice were deficient in HO-1 mRNA and HO-1 protein but not HO-2 mRNA or protein.

HO-1 transgenic mice

Standard techniques were used to generate mice which express a hHO-1 transgene in heart tissue. The transgene was cloned under the control of the cardiac α-myosin heavy chain promoter for expression preferentially in cardiovascular tissue. One group of transgenic mice were engineered to express hHO-1 DNA in the sense orientation, and another group expressed hHO-1 DNA in the antisense orientation. As shown in Fig. 9, hHO-1 mRNA was detected in the heart (ventricle) of the transgenic mouse but not in other tissues tested. Western blot analysis confirmed the presence of a transgenic hHO-1 gene product in heart tissue (ventricles) of hHO-1 transgenic mice. hHO-1 transgenic mice are used to evaluate the effect of HO-1 expression (and overexpression) in cardiovascular tissue, e.g., in response to injury or stress.

Inhibition of cardiomyocyte death

Oxidative stress caused by such conditions as ischemia and reperfusion injury induces myocardial dysfunction and cardiomyocyte death. HO is an enzyme that catalyzes oxidation of heme to generate carbon monoxide (CO; which can increase cellular cGMP) and biliverdin (which is a potent antioxidant). HO-1 is an inducible isoform of HO, whereas HO-2 is constitutively expressed. Expression of HO-1 is induced in the cardiovascular system by such stimuli as hypoxia, hyperoxia, cytokines such as interleukin-1β (IL-1β), endotoxemia, heat shock, and ischemia. HO-1 also regulates VSMC growth. Expression of inducible HO (HO-1)

is markedly induced in the cardiovascular system by stimuli such as increased pressure and hypoxia.

To study the effect of HO-1 on mammalian responses to hypoxia such as that manifested in clinical conditions, e.g., high altitude pulmonary edema, myocardial infarction, myocarditis, pulmonary hypertension, pulmonary embolism, pulmonary valve stenosis, congenital heart disease, and chronic obstructive pulmonary disease (e.g., emphysema), mice were subjected to chronic hypoxia. In accordance with a standard model of pulmonary hypertension, mice were kept in a 10% O_2 chamber for 7 weeks. Two groups (Group I = five HO-1 +/+ mice; Group II = five HO-1 -/- mice) were studied. Two of the HO-1 deficient mice died at week 7; none of the HO-1 +/+ mice died. As shown in Fig. 2, exposure of the mice to hypoxic conditions resulted in an increase in hematocrit in both wild type and knockout (HO-1 -/-) mice, indicating a high level of tissue hypoxia of the mice. Under normoxia conditions, the heart weight of HO-1-deficient and wild type mice was comparable, but under hypoxic conditions the ventricular weight of HO-1-deficient mice greater than that of the HO-1-deficient mice kept under normoxic conditions (Fig. 3). For example, exposure to hypoxia for 7 weeks caused a 32% increase in the ventricular weight index in wild type mice, whereas in HO-1-deficient mice, the ventricular weight index after 7 weeks of hypoxia increased 100% (compared to normoxic wild type mice. Changes in the ventricular weight reflected mainly a right ventricular effect, as the RV(LV+septum) increased in HO-1-deficient mice compared to wild type mice exposed to hypoxia for 7 weeks.

Fig. 7 shows the effect of hypoxia on right ventricular systolic pressure, an indicator of pulmonary arterial systolic pressure. Right ventricular systolic

pressure in wild type and HO-1 -/- mice did not differ under normoxic conditions (P = 0.80; Fig. 7, open bars). Although five weeks of hypoxia increased right ventricular systolic pressure, it did so to a similar degree in wild type and HO-1 -/- mice (P = 0.43; Fig. 7, filled bars).

In HO-1-deficient mice, exposure to conditions of chronic hypoxia resulted in more dramatic hypertrophy and dilation of the right ventricle of the heart compared to that observed in wild type mice. Evidence of massive cardiomyocyte death was detected and large organized thrombi attached to areas of infarct were also detected in HO-1-deficient mice but not in wild type mice.

When stained with Masson's trichrome stain (which detects collagen), an increase in collagen fibers was observed in tissue sections of the right ventricle of HO-1 -/- mice in response to hypoxia compared to the amount of collagen detected in wild type sections. These data indicate evidence of scar formation and repair mechanisms in the hearts of HO-1-deficient mice under hypoxic conditions. Tissue sections were also stained with PECAM stain (which detects endothelial cells). Increased blood vessel formation was detected in the right ventricles of HO-1 -/- mice in response to hypoxia compared to wild type mice. These data suggest that H0-1 inhibits angiogenesis in response to hypoxia.

The mechanism of cardiomyocyte death in HO-1 -/- mice under hypoxic conditions was evaluated by histological analysis, immunocytochemistry, and TdT-mediated dUTP-biotin nickend labeling (TUNEL assay). The standard TUNEL assay detects apoptosis. Ventricles were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or Masson's trichrome. To detect oxidation-specific lipid-protein adducts, heart tissue

sections were immunostained with polyclonal antibody MAL-2 (anti-malondialdehyde-lysine; Rosenfeld et al., 1990, Arteriosclerosis 10:336-349) and counterstained with methyl green. TUNEL was used to detect DNA breaks in apoptotic cells *in situ*. Red staining in the nuclei of the cells indicated a positive reaction; the cells were also counterstained with methyl green. Pulmonary vascular remodeling was assessed in lungs that had been perfused with saline through the pulmonary artery and fixed with 4% paraformaldehyde instilled through the trachea. Muscularization of peripheral vessels was determined using standard methods, e.g., that described in Klinger et al., 1993, J. Appl. Physiol. 75:198-205. The nuclei of cardiomyocytes of HO-1-deficient mice subjected to hypoxic conditions stained positive, providing evidence that apoptosis contributes to the mechanism of death of cardiomyocytes under these conditions.

Additional histological analyses were undertaken to confirm that chronic hypoxia induces right ventricular infarction in HO-1-deficient mice. Cardiomyocytes were intact in ventricular sections from wild type mice exposed to 7 weeks of hypoxia, but ventricular sections from HO-1-deficient mice exposed to 7 weeks of hypoxia showed mononuclear inflammatory cell infiltration, extensive cardiomyocyte degeneration, and death with focal calcification. These observations indicate that infarcts were 1-2 weeks old. The right ventricular infarcts did not appear to result from vascular occlusion, because the coronary arteries supplying blood to the right ventricle were patent in H0-1-deficient mice.

To detect collagen accumulation indicative of fibrosis, ventricular sections were stained with Masson's trichrome. After 7 weeks of hypoxia, cells surrounding

blood vessels stained positive for collagen in hearts from wild type mice. In hearts from HO-1-deficient mice exposed to hypoxia for 7 weeks, early collagen deposition was present throughout the lesion. The degree of fibrosis was consistent with early scar formation and a lesion of 1-2 weeks old.

Two out of the six hearts from 7-week hypoxic HO-1-deficient mice did not exhibit right ventricular infarcts; however, close examination of the hearts revealed focal areas of myocardial degeneration without evidence of extensive inflammatory cell infiltration. These hearts showed early evidence of myocardial damage. Heart from wild type and HO-1-deficient mice examined after 5 weeks of hypoxia displayed no cardiomyocyte degeneration or death and no extensive mononuclear inflammatory cell infiltration. Cells surrounding blood vessels stained positive for collagen in hearts from wild type and HO-1-deficient mice housed for 5 weeks under normoxic and hypoxic conditions. In contrast with the right ventricular free walls from HO-1-deficient mice exposed to 7 weeks of hypoxia (which showed deposition of collagen), no collagen deposition was evident in the right ventricular free walls from HO-1-deficient mice exposed to 5 weeks of hypoxia. These data confirm that in the HO-1-deficient mice exposed to 7 weeks of hypoxia, myocardial infarcts were less than 2 weeks old.

To assess oxidative damage in hearts with infarcts taken from HO-1-deficient mice, ventricular tissue sections were immunostained with MAL-2 which detects oxidation-specific lipid-protein adducts. In contrast to the minimal MAL-2 staining observed in hearts from wild type mice, MAL-2 staining was intense in cells (predominantly cardiomyocytes) beneath the infarcted area of the right ventricle in HO-1-deficient mice. These data indicate the presence of severe oxidative damage

within and around the infarct site. No TUNEL-positive cardiomyocytes were detectable in right ventricles from wild-type mice, but a significant number of TUNEL-positive cells surrounded the infarct site in right ventricles form HO-1-deficient mice.

The data described herein indicate that (1) pulmonary vascular remodeling in response to hypoxia is similar in HO-1 +/+ and -/- mice, (2) hypoxia induces more severe right ventricular hypertrophy in HO-1 -/-mice than in HO+/+ mice, and (3) in HO-1 -/- (but not +/+ mice), massive cardiomyocyte death occurs with large organized thrombi attached to the infarct site. Although some cardiomyocyte death appears to be due to necrosis, apoptosis is a significant mechanism of cardiomyocyte death. Hypoxia and elevated pulmonary arterial pressure increase cardiac production of reactive oxygen species, which play a significant role in myocardial death during ischemia/reperfusion.

Although myocardial infarction has been shown to increase oxidative stress, a 2-3 fold increase in the nitration of protein tyrosine residues (which indicates the presence of the potent oxidant peroxynitrite) was detected in noninfarcted HO-1-deficient hearts exposed to 7 weeks of hypoxia. These data indicate that an increase in oxidative stress precedes gross myocardial infarction.

Evidence of extensive lipid peroxidation in the zone of right ventricular infarction supports the conclusion that the absence of HO-1 in cardiomyocytes leads to an accumulation of reactive oxygen species that causes cardiomyocyte death. Administration of HO-1 or overexpression of HO-1 protects against cardiomyocyte damage from hemodynamic stress or ischemia/reperfusion.

Adaptation of the cardiovascular system to hypoxia

The gene deletion studies described herein indicate that HO-1 plays an important protective role *in*

vivo in the adaptation of the cardiovascular system to hypoxia. Right ventricles from HO-1 -/- mice were severely dilated and contained right ventricular infarcts with mural thrombi.

Humans and animals respond to hypoxia by exhibiting pulmonary vascular remodeling, pulmonary hypertension, and hypertrophy of the right ventricle. The data described herein were obtained using a mouse model of vascular injury which mimics the human response. Hypoxia induces HO-1 expression in the lung, and CO generated by hypoxic VSMCs inhibits proliferation of these cells.

The data described herein indicate that the absence of HO-1 results in a maladaptive response in cardiomyocytes exposed to hypoxia-induced pulmonary hypertension. In the absence of HO-1, VSMC are more sensitive to oxidative stress and have a maladaptive response to pressure overload. HO-1 has a protective effect on cardiomyocytes and VSMC subjected to stress such as pressure-induced injury and secondary oxidative damage.

Therapeutic administration of HO

In the absence of HO-1, cardiomyocytes undergo apoptotic cell death when subjected to stress such as pressure overload or exposure to reactive oxygen species and that death can be inhibited by contacting the cells with HO. For example, HO-1, HO-2, or HO-3 protein or polypeptide (or DNA encoding HO-1, HO-2, or HO-3) is administered locally to heart tissue affected by hypoxic conditions. One means for accomplishing local delivery is providing an HO or DNA encoding and HO on a surface of a vascular catheter, e.g., a balloon catheter coated with an antioxidant, which contacts the wall of the blood vessel to deliver therapeutic compositions at the site of

contact. Drug delivery catheters can also be used to administer solutions of therapeutic compositions.

HO-1 is therapeutically overexpressed (e.g., by administering an inducing agent to increase expression from the endogenous gene) or by administering DNA (alone or in a plasmid) encoding an HO such as HO-1 or HO-2 (or an active fragment thereof, i.e., a fragment has the activity of inhibiting cardiomyocyte death). Inducing agents that stimulate HO-1 expression in cells include hemin, hemoglobin, and heavy metals, e.g., $SnCl_2$ or $NiCl_2$. For example, 250 mmol/kg of body weight of $SnCl_2$ or $NiCl_2$ is administered subcutaneously or 15 mg/kg of body weight of hemin is administered intraperitoneally to laboratory animals. Doses for human patients are determined and optimized using standard methods.

Tables 1 and 3 show human HO-1 and HO-2 cDNA, respectively, in which the polypeptide-encoding nucleotides are designated in bold type and the termination codon is underlined. Tables 2 and 4 show the amino acid sequences of human HO-1 and HO-2, respectively. Tables 5 and 6 show the nucleotide and amino acid sequence of rat HO-3.

TABLE 1: Human HO-1 cDNA

1 tcaacgcctg cctcccctcg agcgtcctca gcgcagccgc cgcccgcgga gccagcacga

61 acgagcccag caccggccgg **atggagcgtc cgcaacccga cagcatgccc caggatttgt**

121 **cagaggccct gaaggaggcc accaaggagg tgcacaccca ggcagagaat gctgagttca**

181 **tgaggaactt tcagaagggc caggtgaccc gagacggctt caagctggtg atggcctccc**

241 **tgtaccacat ctatgtggcc ctggaggagg agattgagcg caacaaggag agcccagtct**

301 **tcgcccctgt ctacttccca gaagagctgc accgcaaggc tgccctggag caggacctgg**

361 **ccttctggta cgggccccgc tggcaggagg tcatccccta cacaccagcc atgcagcgct**

421 **atgtgaagcg gctccacgag gtggggcgca cagagcccga gctgctggtg gcccacgcct**

481 **acacccgcta cctgggtgac ctgtctgggg gccaggtgct caaaaagatt gcccagaaag**

541 **ccctggacct gcccagctct ggcgagggcc tggccttctt caccttcccc aacattgcca**

601 **gtgccaccaa gttcaagcag ctctaccgct cccgcatgaa ctccctggag atgactcccg**

661 **cagtcaggca gagggtgata gaagaggcca agactgcgtt cctgctcaac atccagctct**

721 **ttgaggagtt gcaggagctg ctgacccatg acaccaagga ccagagcccc tcacgggcac**

781 **cagggcttcg ccagcgggcc agcaacaaag tgcaagattc tgcccccgtg gagactccca**

841 **gagggaagcc cccactcaac acccgctccc aggctccgct tctccgatgg gtccttacac**

901 **tcagctttct ggtggcgaca gttgctgtag ggctttatgc catgtgaatg caggcatgct**

961 ggctcccagg gccatgaact ttgtccggtg gaaggccttc tttctagaga gggaattctc
1021 ttggctggct tccttaccgt gggcactgaa ggctttcagg gcctccagcc ctctcactgt
1081 gtccctctct ctggaaagga ggaaggagcc tatggcatct tccccaacga aaagcacatc
1141 caggcaatgg cctaaacttc agaggggggcg aaggggtcag ccctgccctt cagcatcctc
1201 agttcctgca gcagagcctg gaagacaccc taatgtggca gctgtctcaa acctccaaaa
1261 gccctgagtt tcaagtatcc ttgttgacac ggccatgacc actttccccg tgggccatgg
1321 caatttttac acaaacctga aaagatgttg tgtcttgtgt ttttgtctta tttttgttgg
1381 agccactctg ttcctggctc agcctcaaat gcagtatttt tgttgtgttc tgttgttttt
1441 atagcagggt tggggtggtt tttgagccat gcgtgggtgg ggagggaggt gtttaacggc
1501 actgtggcct tggtctaact tttgtgtgaa ataataaaca acattgtctg
(SEQ ID NO:1)

Table 2: Human HO-1 amino acid sequence

MERPQPDSMP QDLSEALKEA TKEVHTQAEN AEFMRNFQKG QVTRDGFKLV MASLYHIYVA
LEEEIERNKE SPVFAPVYFP EELHRKAALE QDLAFWYGPR WQEVIPYTPA MQRYVKRLHE
VGRTEPELLV AHAYTRYLGD LSGGQVLKKI AQKALDLPSS GEGLAFFTFP NIASATKFKQ
LYRSRMNSLE MTPAVRQRVI EEAKTAFLLN IQLFEELQEL LTHDTKDQSP SRAPGLRQRA
SNKVQDSAPV ETPRGKPPLN TRSQAPLLRW VLTLSFLVAT VAVGLYAM (SEQ ID NO:2)

Table 3: Human HO-2 cDNA

1 gggctgactg gaggctggcg gacaggcgac agacctgcgg caggaccaga ggagcgagac

61 gagcaagaac cacacccagc agcaatgtca gcggaagtgg aaacctcaga gggggtagac

121 gagtcagaaa aaaagaactc tggggcccta gaaaaggaga accaaatgag aatggctgac

181 ctctcagagc tcctgaagga agggaccaag gaagcacacg accgggcaga aaacacccag

241 tttgtcaagg acttcttgaa aggcaacatt aagaaggagc tgtttaagct ggccaccacg

301 gcactttact tcacatactc agccctcgag gaggaaatgg agcgcaacaa ggaccatcca

361 gcctttgccc ctttgtactt ccccatggag ctgcaccgga aggaggcgct gaccaaggac

421 atggagtatt tctttggtga aaactgggag gagcaggtgc agtgccccaa ggctgcccag

481 aagtacgtgg agcggatcca ctacataggg cagaacgagc cggagctact ggtggcccat

541 gcatacaccc gctacatggg ggatctctcg gggggccagg tgctgaagaa ggtggcccag

601 cgagcactga aactccccag cacaggggaa gggacccagt tctacctgtt tgagaatgtg

661 gacaatgccc agcagttcaa gcagctctac cgggccagga tgaacgccct ggacctgaac

721 atgaagacca aagagaggat cgtggaggcc aacaaggctt ttgagtataa catgcagata

781 ttcaatgaac tggaccaggc cggctccaca ctggccagag agaccttgga ggatgggttc

841 cctgtacacg atgggaaagg agacatgcgt aaatgccctt tctacgctgc tgaacaagac

901 aaagggctgg agggcagcct gtcccttccg acaagctatg ctgtgctgag gaagcccagc

961 ctccagttca tcctggccgc tggtgtggcc ctagctgctg gactcttggc ctggtactac

1021 atgtgaagca cccatcatgc cacaccggta ccctcctccc gactgaccac tggcctaccc

1081 ctttctccag ccctgactaa actaccacct caggtgactt tttaaaaaat gctgggttta

1141 agaaaggcaa ccaataaaag agatgctaga gcctcgtctg acagcatcct ctctatgggc

1201 catattccgc actgggcaca ggccgtcacc ctgggagcag tcggcacagt gcagcaagcc

1261 tggcccccga cccagctcta ctccaggctt ccacacttct gggccctagg ctgcttccgg

1321 tagtccctgt ttttgcagta catgggtgac tatctccct gttggaggtg agtggcctgt

1381 aagtccaagc tgtgcgaggg ggccttgctg gatgctgctg tacaacttct gggcctctct

1441 tggaccctgg gagtgagggt gggtgtgggt ggaagcctca gaggccttgg gagctcatcc

1501 ctctcaccca gaatccctct aacccttggg tgcggtttgc tcagccccag cttatctcct

1561 cctccgcctg tgtaaatgct ccagcactca ataaagtggg ctttgcaagc taaaaaaaaa

1621 aaaaaaa (SEQ ID NO:3)

Table 4: Human HO-2 amino acid sequence

MSAEVETSEGVDESEKKNSGALEKENQMRMADLSELLKEGTKEAHDRAENTQFVKDF
LKGNIKKELFKLATTALYFTYSALEEEMERNKDHPAFAPLYFPMELHRKEALTKDME
YFFGENWEEQVQCPKAAQKYVERIHYIGQNEPELLVAHAYTRYMGDLSGGQVLKKVA
QRALKLPSTGEGTQFYLFENVDNAQQFKQLYRARMNALDLNMKTKERIVEANKAFEY
NMQIFNELDQAGSTLARETLEDGFPVHDGKGDMRKCPFYAAEQDKGLEGSLSLPTSY
AVLRKPSLQFILAAGVALAAGLLAWYYM (SEQ ID NO:4)

Table 5: Rat HO-3 nucleotide sequence

1 tttcagggat ttttgcgatt cctctctgta gacttctact
tgttctctaa gggagttctt
61 catgtctttc ttgaagtcat ccagcatcat gatcaaatat
gattttgaaa ctagatcttg
121 cttttctggt gtgtttggat attccatgtt tgttttggtg
ggagaattgg gctccgatga
181 tggcatgtag tcttggtttc tgttgcttgg tttcctgcgc
ttgcctctcg ccatcagatt
241 atctctagtg ttactttgtt ctgctatttc tgacagtggc
tagactgtcc tataagcctg
301 tgtgtcagga gtgctgtaga cctttttttcc tctctttcag
tcagttatgg gacagagtgt
361 tctgcttttg ggcgtgtagt ttttcctctc tacaggtctt
cagctgttcc tgtgggcctg
421 tgtcttgagt tcaccaggca gctttcttgc agcagaaaat
ttggtcatac ctgtgatcct
481 gaggctcaag ttcgctcgtg gggtgctgtc caggggctct
ctgcagcggg cacaaccagg
541 aagacctgtg cggcccttc cggagcttca gtgcaccagg
gttccagatg gcctttggcg
601 ttttcctctg gcgtccgaga tgtatgtaca gagagcagtc
tcttctggtt tcccaggctt
661 gtctgcctct ctgaaggttc agctctccct cccacgggat
ttgggtgcag agaactgttt

721 atccggtctg tttctttcag gttccggtgg tgtctcaggc aggtgtcgtt cctgcgccct

781 cccccatggg accagaggcc ttatacagtt tcctcttggg ccagggatgt gggcaggggt

841 gagcagtgtt ggtggtctct tccgtctgca gcctcaggag tgccacctga ccaggcggtt

901 gggtctctct ctgagaattt cattttaaa tcattcatta aaatgtcatg acttgatgtc

961 ctgctgtccg tctcacgccc tcagctgtaa cagtgccgag ggagtcactg aagaagagac

1021 tgaatgacca gagtatgggc agcacagaca actcaacaaa aatgtcttca gaggtggaga

1081 ctgcggaggc cgtagatgag tcagagaaga actctatggc atcagagaag gaaaaccatt

1141 ccaaaatagc agacttttct gatcttctga aggaagggac aaaggaagca gatgaccggg

1201 cagaaaatac ccagtttgtc aaagacttct tgaaaggaaa cattaagaag gagctattta

1261 agctggccac cactgcactt tcatactcag cccctgagga ggaaatggat tcactgacca

1321 aggacatgga gtacttcttt ggtgaaaact gggaggaaaa agtgaagtgc tctgaagctg

1381 cccagacgta tgtggatcag attcactatg tagggcaaaa tgagccagag catctggtgg

1441 cccatactta ctctacttac atgggggaa acctttcagg ggaccaggta ctgaagaagg

1501 agacccagcc ggtccccttc actagggaag ggactcagtt ctacctgttt gagcatgtag

1561 acaatgctaa gcaattcaag ctattctact gcgctagatt gaatgccttg gacctgaatt

1621 tgaagaccaa agagaggatt gtggaggaag ccaccaaagc ctttgaatat aatatgcaga

1681 tattcagtga actggaccag gcaggctcca taccagtaag agaaacccta aagaatgggc

1741 tctcaatact tgatgggaag ggaggtgtat gcaaatgtcc
ctttaatgct gctcagccag
1801 acaaaggtac cctgggaggc agcaactgcc ctttccagat
gtccatggcc ttgctgagga
1861 agcctaactt gcagctcatt ctagttgcca gtatggcctt
ggtagctgga cttttagcct
1921 ggtactacat gtgaagggcc tgtcaagttg tttgcatcct
atctcaacat cctaccactt
1981 gttccttccc cacctccacc tctgcctaga actaccacct
caggtgacat ttttaatgtt
2041 gggtttgaga aaatgagcaa ccaataaaag acagacccta
gaaaaaagtc atgacttaag
2101 tggcacgggg acacctaaag tcacactttg tgcttcagac
atactttctt tctctatttc
2161 aacactgaat tcgggaagta acctactact attaataata
aatgctacac aatgcataat
2221 aaaaa (SEQ ID NO:5)

Table 6: Rat HO-3 amino acid sequence

MSSEVETAEAVDESEKNSMASEKENHSKIADFSDLLKEGTKEADDRAENTQFVKDFL
KGNIKKELFKLATTALSYSAPEEEMDSLTKDMEYFFGENWEEKVKCSEAAQTYVDQI
HYVGQNEPEHLVAHTYSTYMGGNLSGDQVLKKETQPVPFTREGTQFYLFEHVDNAKQ
FKLFYCARLNALDLNLKTKERIVEEATKAFEYNMQIFSELDQAGSIPVRETLKNGLS
ILDGKGGVCKCPFNAAQPDKGTLGGSNCPFQMSMALLRKPNLQLILVASMALVAGLL
AWYYM (SEQ ID NO:6)

An HO preferably has an amino acid sequence that is at least 85% identical (preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably at least 100% identical) to the amino acid sequence of SEQ ID NO: 2, 4, or 6. DNA encoding an HO preferably has nucleotide sequence that is at least 50% identical (preferably at least 75%, more preferably at least 85%, more preferably at least 95%, most

preferably at least 100% identical) to the nucleotide sequence of the coding region of SEQ ID NO:1, 3, or 5. The per cent identity of nucleotide and amino acid sequences is determined using the Sequence Analysis Software Package developed by the Genetics Computer Group (University of Wisconsin Biotechnology Center, Madison, WI), employing the default parameters thereof.

To be clinically beneficial, expression of HO from an endogenous gene or expression of recombinant HO from exogenous DNA need not be long term. For example, to inhibit cardiomyocyte death associated with a myocardial infarction or other acute condition which results in oxidative stress to the tissue, the most critical period of treatment is the first three months after injury. Thus, gene therapy to express recombinant HO for even a period of days or weeks after administration of the HO-encoding DNA (which administration is prior to or soon after an injury) minimizes cell death, inhibits VSMC proliferation, and therefore confers a clinical benefit.

For local administration of DNA to cardiovascular tissue, standard gene therapy vectors used. Such vectors include viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO 89/07136; Rosenberg et al., 1990, N. Eng. J. Med. 323(9):570-578), adenovirus (see, e.g., Morsey et al., 1993, J. Cell. Biochem., Supp. 17E,), adeno-associated virus (Kotin et al., 1990, Proc. Natl. Acad. Sci. USA 87:2211-2215,), replication defective herpes simplex viruses (HSV; Lu et al., 1992, Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sept. 22-26, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), and any modified versions of these vectors. The invention may utilize any other delivery system which accomplishes *in vivo* transfer of nucleic acids into eukaryotic cells. For example, the

nucleic acids may be packaged into liposomes, e.g., cationic liposomes (Lipofectin), receptor-mediated delivery systems, non-viral nucleic acid-based vectors, erythrocyte ghosts, or microspheres (e.g., microparticles; see, e.g., U.S. Patent No. 4,789,734; U.S. Patent No. 4,925,673; U.S. Patent No. 3,625,214; Gregoriadis, 1979, Drug Carriers in Biology and Medicine, pp. 287-341 (Academic Press,). Naked DNA may also be administered. Alternatively, a plasmid which directs cardiospecific expression (e.g., a plasmid containing a myosin heavy chain (αMHC) promoter; Fig. 6) of an HO-encoding sequence can be used for gene therapy. Expression of an HO (encoded, e.g., by the coding sequences of SEQ ID NO:1, 3, or 5) from such a constitutive promoter is useful to inhibit cardiomyocyte death *in vivo*. Nucleic acids which hybridize at high stringency to the coding sequences of SEQ ID NO:1, 3, or 5 and which encode a polypeptide which has a biological activity of an HO polypeptide (e.g., inhibition of cardiomyocyte death) are also used for gene therapy for vascular injury. To determine whether a nucleic acid hybridizes to a reference nucleic acid at a given stringency, hybridization is carried out using standard techniques, such as those described in Ausubel *et al.* (*Current Protocols in Molecular Biology*, John Wiley & Sons, 1989). "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration, *e.g.*, wash conditions of 65°C at a salt concentration of approximately 0.1 X SSC. "Low" to "moderate" stringency refers to DNA hybridization and wash conditions characterized by low temperature and high salt concentration, *e.g.*, wash conditions of less than 60°C at a salt concentration of at least 1.0 X SSC. For example, high stringency conditions may include hybridization at

about 42°C, and about 50% formamide; a first wash at about 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% x SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to an CHF-1 gene are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at about 42°C, about 6X SSC, and about 1% SDS; and a second wash at about 50°C, about 6X SSC, and about 1% SDS. To determine whether a polypeptide encoded by a hybridizing nucleic acid has a biological activity of an HO polypeptide, the polypeptide is evaluated using any of the functional assays to measure HO activity described herein, e.g., measuring VSMC proliferation or cardiomyocyte death.

For gene therapy of cardiovascular tissue, fusigenic viral liposome delivery systems known in the art (e.g., hemagglutinating virus of Japan (HVJ) liposomes or Sendai virus-liposomes) are useful for efficiency of plasmid DNA transfer (Dzau et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:11421-11425). Using HVJ-liposomes, genes are expressed from plasmid DNA delivered to target tissues *in vivo* for extended periods of time (e.g., greater than two weeks for heart and arterial tissue and up to several months in other tissues).

DNA for gene therapy can be administered to patients parenterally, e.g., intravenously, subcutaneously, intramuscularly, and intraperitoneally. Sustained release administration such as depot injections or erodible implants, e.g., vascular stents coated with DNA encoding an HO, may also be used. The compounds may also be directly applied during surgery, e.g, bypass surgery, or during angioplasty, e.g, an angioplasty catheter may be coated with DNA encoding an HO. The DNA

is then deposited at the site of angioplasty. DNA or an inducing agent is administered in a pharmaceutically acceptable carrier, i.e., a biologically compatible vehicle which is suitable for administration to an animal e.g., physiological saline. A therapeutically effective amount is an amount which is capable of producing a medically desirable result, e.g., expression of HO, in a treated animal. Such an amount can be determined by one of ordinary skill in the art. As is well known in the medical arts, dosage for any given patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, severity of arteriosclerosis or vascular injury, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for intravenous administration of DNA is approximately 10^6 to 10^{22} copies of the DNA molecule.

HO-based therapy for cardiovascular disorders depends on when (in the course of a vascular injury) the patient is encountered. HO-1 -/- VSMC initially proliferated at a faster rate compared to wild type VSMC within days after an insult. Increasing local HO-1 levels at this stage inhibits VSMC growth and confers a clinical benefit. For example, if the patient is encountered at an early stage (e.g., within one week of cardiovascular stress or injury), the patient is treated by augmenting the local level of HO-1 (e.g., by administering an HO polypeptide, by increasing expression of endogenous HO, or by standard gene therapy techniques described above to produce recombinant HO *in vivo*) to inhibit the growth of VSMC and decrease the size of a myocardial infarct. In contrast, if the patient is encountered at a later stage (e.g., several weeks, 1 month, 2 months, and up to 3 months after an injury), the

patient is treated by inhibiting HO expression to decrease formation of a stenotic lesion, e.g., by antisense therapy, as described below.

Organ and tissue preservation

Concerns about irreversible ischemic tissue damage arise when a donor organ, e.g., a heart, is removed from the donor and stored for more than about 5-6 hours before transplantation into the recipient. *Ex vivo* treatment of a donor organ to reduce tissue damage by inhibiting death of cardiomyocytes is carried out by immersing the organ in a solution containing an inducing agent, an HO, e.g, HO-1 or HO-2, or a nucleic acid encoding an HO prior to transplantation. By "ex vivo treatment" is meant treatment that takes place outside of the body. For example, *ex vivo* treatment is administered to an organ (or a tissue fragment or dissociated cells) that has been removed from a mammal and which will be returned to the same or a different mammal (at the same or different anatomical site) after the treatment. For example, effective DNA delivery to cells in solid organs achieved by contacting the organ with a combination of DNA, liposomes and transferrin during the cold ischemic time prior to transplantation (see, e.g., Hein et al. 1998, Eur. J. Cardiothorac. Surg. 13:460-466). An organ may also be perfused or electroporated with solution containing HO-encoding DNA. Vectors and delivery systems described above for gene therapy applications are suitable for organ preservation and cell preservation *in vitro*.

Inhibition of restenosis

VSMC proliferation contributes to graft stenosis and restenosis following vascular injury such as that resulting from coronary angioplasty and coronary bypass surgery. Patients with restenosis have a significantly

poorer clinical outcome compared to patients without restenosis.

Using a mouse model of vascular graft stenosis in which the stenosis develops rapidly and closely mimics the development of vascular graft stenosis in humans, the effect of HO-1 on VSMC proliferation was examined. A patch of jugular vein was grafted onto a carotid artery in normal and HO-1 deficient mice to create composite vessels that mimic vein grafts used for bypass surgery (Fig. 4). The vein patch is subject to increased pressure which leads to an increase in local VSMC proliferation and occlusion of the blood vessel (Figs. 5A-B). In wild type mice, there was robust formation of a neointima (characterized by proliferating VSMC) in the vein graft. In contrast, tissue sections of the neointima of HO-1 -/- mice revealed a necrotic mass. The HO-1 -/- neointima was a complex lesion characterized by mostly acellular material, indicating death of VSMC. HO-1 -/- VSMC are more susceptible to H_2O_2-induced death compared to VSMC isolated from wild type mice (Fig. 8). These data indicate that HO-1 is required for VSMC proliferation at later stages post-injury and that local inhibition of HO-1 expression, e.g., by antisense therapy, is useful to inhibit graft stenosis or restenosis in patients affected or who at risk of developing such conditions.

The data described herein indicate that (1) in response to increased pressure, VSMC proliferate in the neointima of the venous patch in HO-1 +/+ mice, and (2) in contrast, massive cell death occurs in the neointima of the venous patch in HO-1 -/- mice.

Patients undergoing invasive vascular procedures, e.g., balloon angioplasty, are at risk for developing undesired vascular stenosis or restenosis. Angioplasty, used to treat arteriosclerosis, involves the insertion of

catheters, e.g., balloon catheters, through an occluded region of a blood vessel in order to expand it. However, the aftermath of angioplasty may be problematic. Restenosis, or closing of the vessel, can occur as a consequence of injury, e.g., mechanical abrasion associated with the angioplasty treatment. This restenosis is caused by proliferation of smooth muscle cells stimulated by vascular injury. Other anatomical disruptions or mechanical disturbances of a blood vessel, e.g., laser angioplasty, coronary artery surgery, atherectomy, coronary artery stents, and coronary bypass surgery, may also cause vascular injury and subsequent proliferation of smooth muscle cells.

Therapeutic approaches, such as antisense therapy or ribozyme therapy are used to inhibit HO expression, and as a result, VSMC proliferation that leads to neointimal thickening. The antisense strand (either RNA or DNA) is directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. Alternatively, a vector-containing sequence which, which once within the target cells is transcribed into the appropriate antisense mRNA, may be administered. Nucleic acids complementary to all or part of the HO cDNA (SEQ ID NO: 1, 3, or 5) may be used in methods of antisense treatment to inhibit expression of HO. Antisense treatment is carried out by administering to a mammal, such as a human, DNA containing a promoter, e.g., a cardiospecific promoter, operably linked to a DNA sequence (an antisense template), which is transcribed into an antisense RNA. By "operably linked" is meant that a coding sequence and a regulatory sequence(s) (*i.e.*, a promoter) are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the regulatory sequence(s). Alternatively, as mentioned

above, antisense oligonucleotides may be introduced directly into vascular cells. The antisense oligonucleotide may be a short nucleotide sequence (generally at least 10, preferably at least 14, more preferably at least 20 (e.g., at least 30), and up to 100 or more nucleotides) formulated to be complementary to a portion, e.g., the coding sequence, or all of HO mRNA. Oligonucleotides complementary to various portions of HO-1 or HO-2 mRNA can readily be tested *in vitro* for their ability to decrease production of HO in cells, using standard methods. Sequences which decrease production of HO message in *in vitro* cell-based or cell-free assays can then be tested *in vivo* in rats or mice to determine whether HO expression (or VSMC proliferation) is decreased.

Ribozyme therapy can also be used to inhibit gene expression. Ribozymes bind to specific mRNA and then cut it at a predetermined cleavage point, thereby destroying the transcript. These RNA molecules may be used to inhibit expression of a gene encoding a protein involved in the formation of vein graft stenosis according to methods known in the art (Sullivan et al., 1994, J. Invest. Derm. 103:85S-89S; Czubayko et al., 1994, J. Biol. Chem. 269:21358-21363; Mahieu et al, 1994, Blood 84:3758-65; Kobayashi et al. 1994, Cancer Res. 54:1271-1275).

Standard methods of administering antisense therapy have been described (see, e.g., Melani et al., 1991, Cancer Res. 51:2897-2901). Antisense nucleic acids which hybridize to HO-encoding mRNA can decrease or inhibit production of HO by associating with the normally single-stranded mRNA transcript, thereby interfering with translation and thus, expression of HO. Such nucleic acids are introduced into target cells by standard vectors and/or gene delivery systems such as those

described above for gene therapy. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses and adeno-associated viruses, among others. For example, antisense oligodesoxynucleotides, e.g., oligonucleotides which have been modified to phosphorthioates or phosphoamidates, withstand degradation after delivery and have been successfully used to inhibit gene expression in a model of reperfusion injury (see, e.g., Haller et al., 1998, Kidney Int. 53:1550-1558). Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal: e.g., physiological saline. A therapeutically effective amount of a compound is an amount which is capable of producing a medically desirable result in a treated animal, e.g., inhibition of expression of HO-1 or a decrease in VSMC proliferation.

Compositions that inhibit HO activity, e.g., its role in the promotion of VSMC proliferation, are also administered to inhibit VSMC-mediated stenosis or restenosis. For example, metalloporphyrins, e.g., zinc protoporphyrin IX (ZnPP), zinc mesoporphyrin IX (ZnMP), tin protoporphyrin IX (SnPP), tin mesoporphyrin IX (SnMP), zinc deuteroporphyrin IX 2,4 bis glycol (ZnDPBG), chromium protoporphyrin (CrPP), cobalt protoporphyrin (CoPP), and manganese metalloporphyrin (MnPP) are administered to mammals at μmol/kg doses to inhibit HO activity. SnPP has safely been administered to human infants at doses of 0.5 μmol/kg to 100μmol/kg of body weight. HO-inhibitory doses for local administration are determined using methods known in the art.

Parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal delivery routes, may be used to deliver the compounds that inhibit

HO activity or expression, with local vascular administration being the preferred route. Dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. For antisense therapy, a preferred dosage for administration of nucleic acids is from approximately 10^6 to 10^{22} copies of the nucleic acid molecule. As described above, local administration to a site of vascular injury or to cardiac tissue is accomplished using a catheter or indwelling vascular stent.

Other embodiments are within the following claims.

What is claimed is:

1. A method of inhibiting cardiomyocyte death in a mammal comprising locally administering to the myocardium of said mammal a heme oxygenase (HO).

2. The method of claim 1, wherein said mammal has suffered a myocardial infarction.

3. The method of claim 1, wherein said mammal has myocarditis.

4. The method of claim 1, wherein said HO is heme oxygenase-1 (HO-1).

5. The method of claim 1, wherein said HO is heme oxygenase-2 (HO-2).

6. A method of inhibiting cardiomyocyte death in a mammal comprising locally administering to the myocardium of said mammal a DNA encoding a HO.

7. The method of claim 6, wherein said HO is HO-1.

8. The method of claim 6, wherein said HO is HO-2 or HO-3.

9. A method of inhibiting cardiomyocyte death *in vitro*, comprising contacting cardiomyocytes with an HO.

10. A method of inhibiting cardiomyocyte death *in vitro*, comprising contacting cardiomyocytes with DNA encoding an HO.

11. The method of claim 10, wherein said HO is HO-1.

12. The method of claim 10, wherein said HO is HO-2.

13. A method of preserving isolated myocardial tissue comprising perfusing said tissue with a solution comprising an HO or a DNA encoding an HO.

14. A method of inhibiting vascular restenosis in a mammal comprising locally administering to the site of a vascular injury a compound which inhibits expression of HO-1.

15. The method of claim 14, wherein said compound inhibits HO-1 transcription in a vascular cell of said mammal.

16. The method of claim 15, wherein said vascular cell is an aortic smooth muscle cell.

17. The method of claim 14, wherein said mammal is a human.

18. The method of claim 14, wherein said compound inhibits translation of HO-1 mRNA in a vascular cell of said mammal.

19. The method of claim 18, wherein said compound consists of a single stranded nucleic acid complementary to at least a portion of said HO-1 mRNA.

20. A method of inhibiting vascular restenosis in a mammal comprising identifying a mammal having vascular restenosis or at risk of developing vascular restenosis and administering to said mammal a compound which inhibits expression of HO-1.

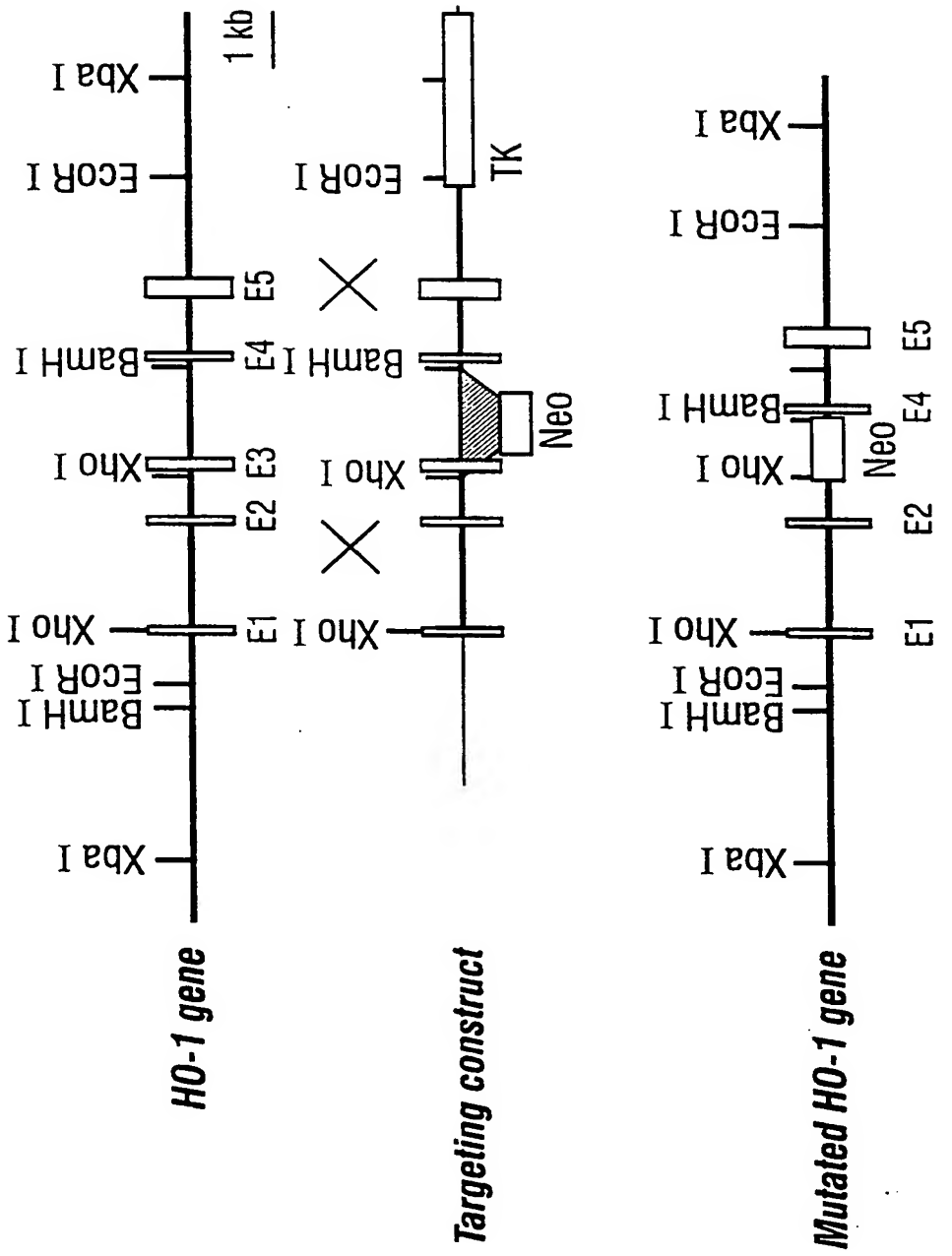
21. The method of claim 14, wherein said compound is administered to said mammal at least one month after a vascular injury.

22. The method of claim 14, wherein said compound is administered to said mammal at least two months after a vascular injury.

23. The method of claim 14, wherein said compound is administered to said mammal at least three months after a vascular injury.

24. A method of inhibiting vascular smooth muscle cell proliferation in a mammal comprising administering to an injured vascular tissue of said mammal a HO, wherein said HO is administered within 24 hours after a vascular injury.

25. The method of claim 24, wherein said HO is administered to said mammal for up to one week after a vascular injury.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EcoR I
E5
BamH I
E4
Xho I
E3
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Xho I
E1
EcoR I
BamH I
Xba I
HO-1 gene
EcoR I
TK
BamH I
Neo
Xho I
Xho I
Targeting construct
Xba I
EcoR I
E5
BamH I
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EcoR I
BamH I
Xba I
Mutated HO-1 gene

FIG. 1

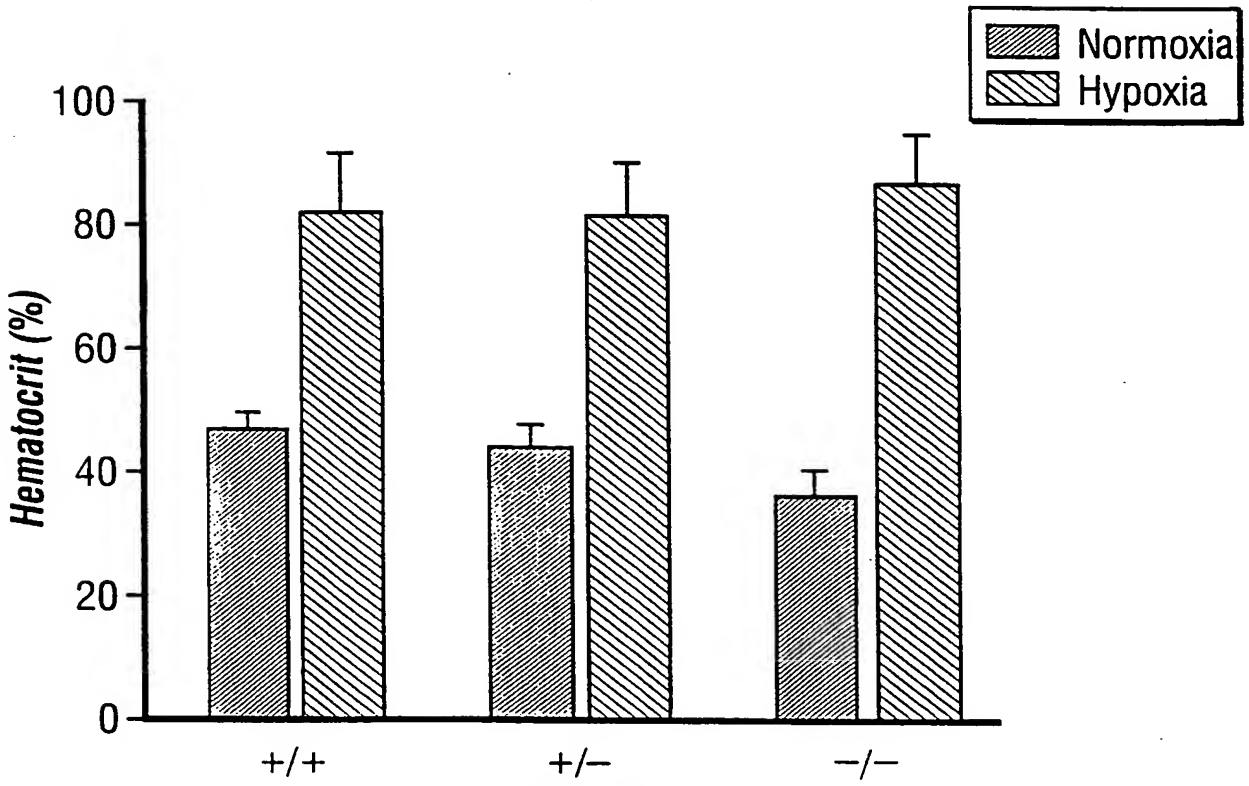
Normoxia
Hypoxia
Hematocrit (%)
100
80
60
40
20
0
+/+
+/–
–/–

FIG. 2

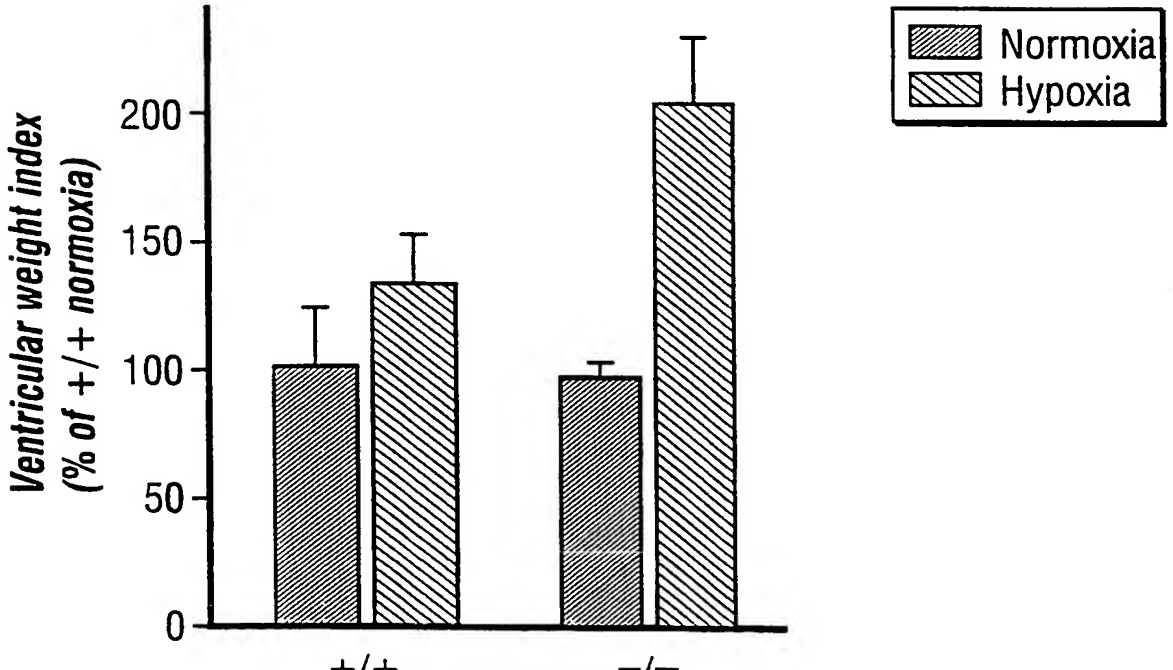
Normoxia
Hypoxia
Ventricular weight index
(% of +/+ normoxia)
200
150
100
50
0
+/+
–/–

FIG. 3

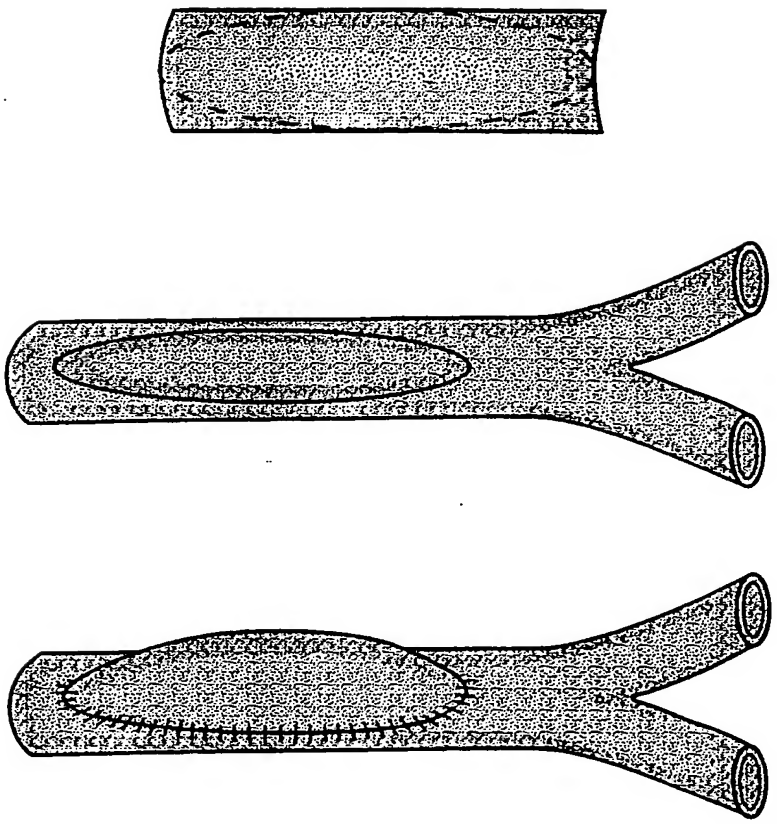
Harvest autogenous vein patch
Remove part of carotid artery wall to produce a defect
Suture venous patch in place

FIG. 4

FIG. 5A

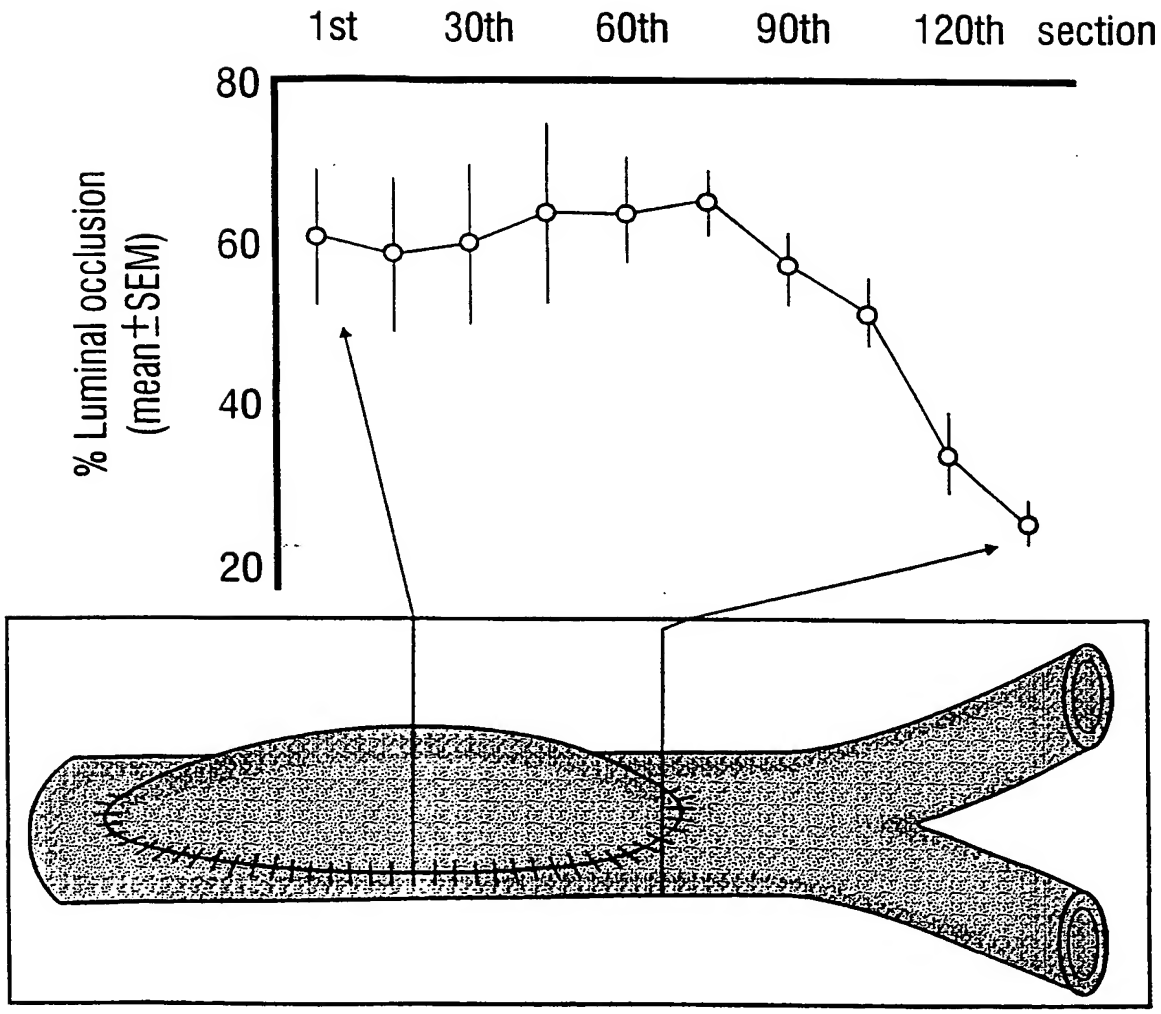
1st
30th
60th
90th
120th section
80
60
40
20
% Luminal occlusion
(mean±SEM)

FIG. 5B

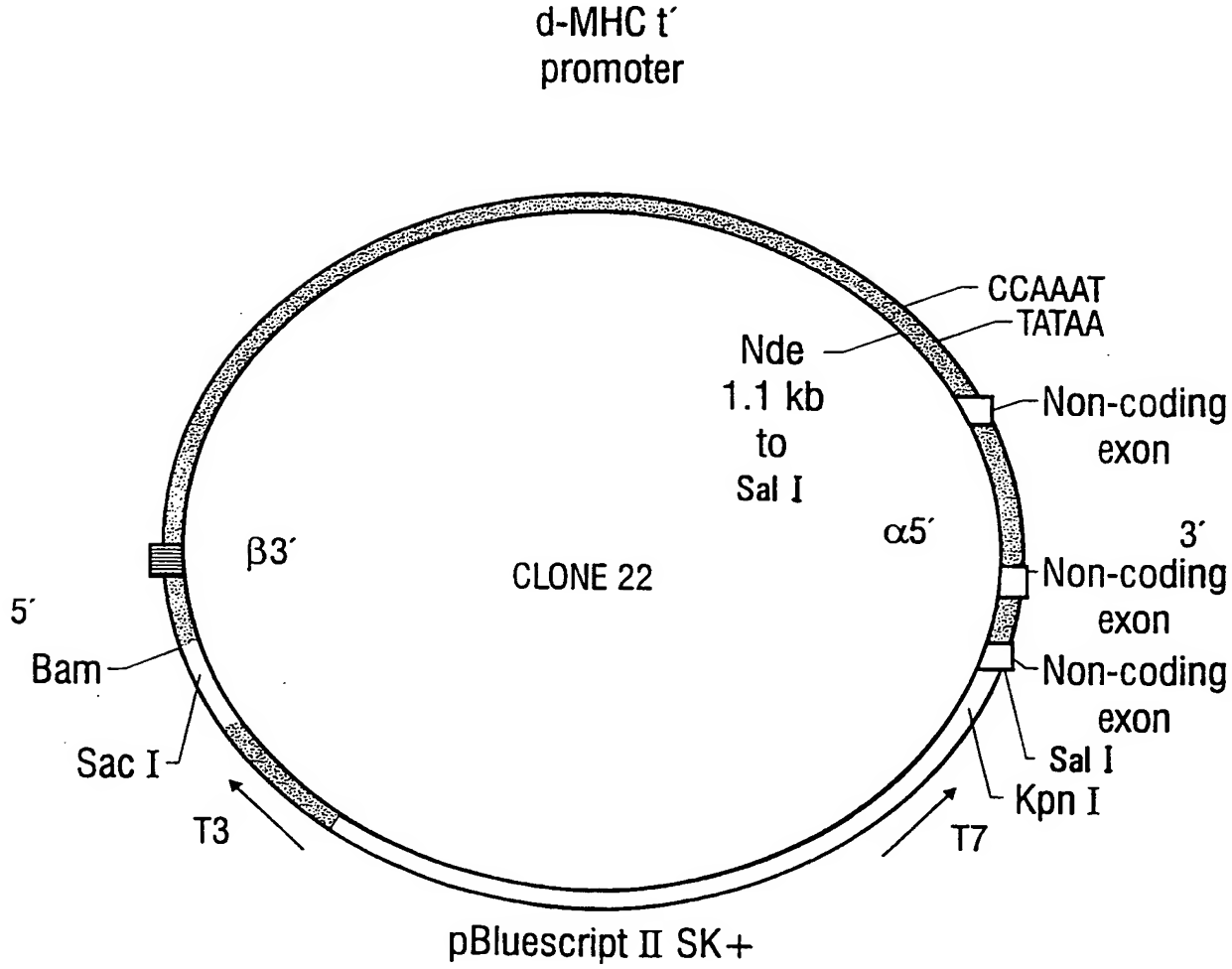
d-MHC t´
promoter
CCAAAT
TATAA
Nde
1.1 kb
to
Sal I
Non-coding
exon
β3´
CLONE 22
α5´
3´
Non-coding
exon
Non-coding
exon
5´
Bam
Sac I
T3
Sal I
Kpn I
T7
pBluescript II SK+

FIG. 6

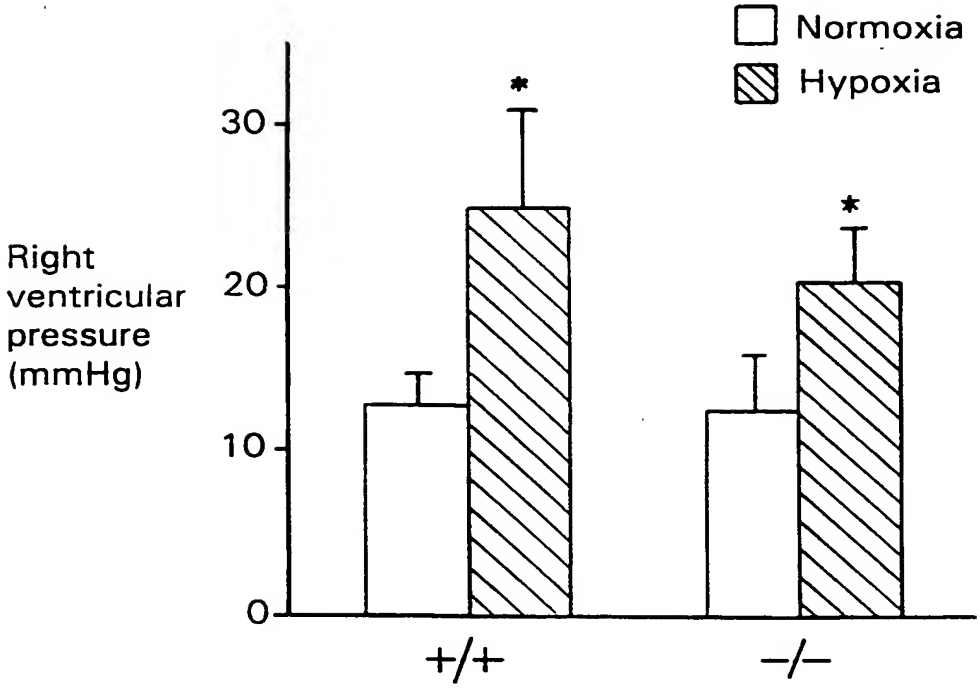
Normoxia
Hypoxia
Right ventricular pressure (mmHg)
30
20
10
0
*
*
+/+
−/−

FIG. 7

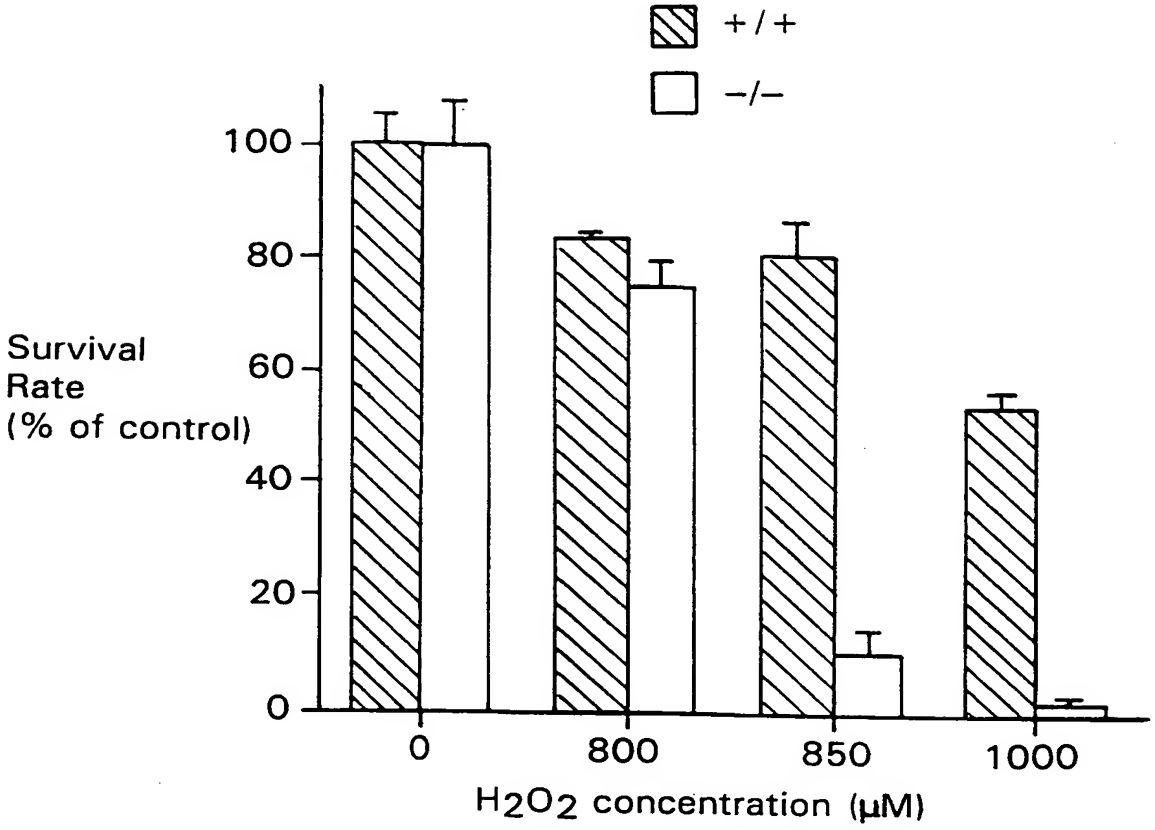
+/+
−/−
Survival Rate (% of control)
100
80
60
40
20
0
0
800
850
1000
H2O2 concentration (μM)

FIG. 8

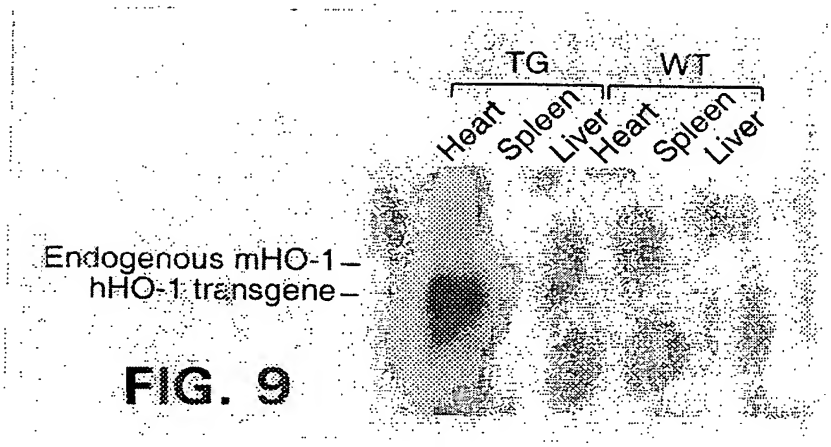
TG
WT
Heart
Spleen
Liver
Heart
Spleen
Liver
Endogenous mHO-1
hHO-1 transgene

FIG. 9

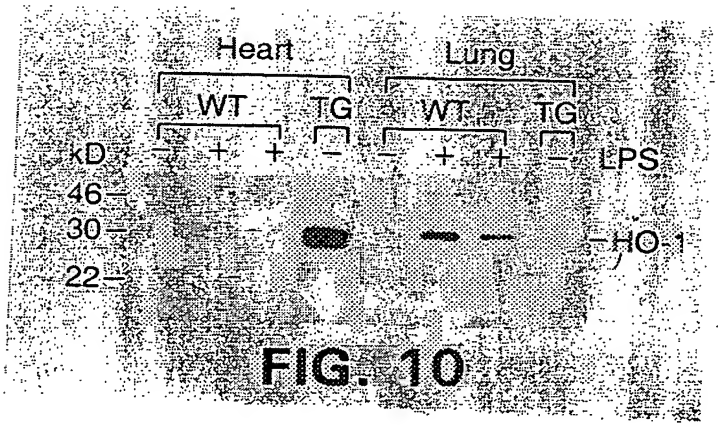
Heart
Lung
WT
TG
WT
TG
kD
LPS
46
30
22
HO-1

FIG. 10

SEQUENCE LISTING

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                        Met Glu Arg Pro Gln Pro Asp Ser Met Pro Gln
                          1               5                  10

gat ttg tca gag gcc ctg aag gag gcc acc aag gag gtg cac acc cag      161
Asp Leu Ser Glu Ala Leu Lys Glu Ala Thr Lys Glu Val His Thr Gln
             15                  20                  25

gca gag aat gct gag ttc atg agg aac ttt cag aag ggc cag gtg acc      209
Ala Glu Asn Ala Glu Phe Met Arg Asn Phe Gln Lys Gly Gln Val Thr
         30                  35                  40

cga gac ggc ttc aag ctg gtg atg gcc tcc ctg tac cac atc tat gtg      257
Arg Asp Gly Phe Lys Leu Val Met Ala Ser Leu Tyr His Ile Tyr Val
     45                  50                  55

gcc ctg gag gag gag att gag cgc aac aag gag agc cca gtc ttc gcc      305
Ala Leu Glu Glu Glu Ile Glu Arg Asn Lys Glu Ser Pro Val Phe Ala
 60                  65                  70                  75

cct gtc tac ttc cca gaa gag ctg cac cgc aag gct gcc ctg gag cag      353
Pro Val Tyr Phe Pro Glu Glu Leu His Arg Lys Ala Ala Leu Glu Gln
                 80                  85                  90

gac ctg gcc ttc tgg tac ggg ccc cgc tgg cag gag gtc atc ccc tac      401
Asp Leu Ala Phe Trp Tyr Gly Pro Arg Trp Gln Glu Val Ile Pro Tyr
             95                  100                 105

aca cca gcc atg cag cgc tat gtg aag cgg ctc cac gag gtg ggg cgc      449
Thr Pro Ala Met Gln Arg Tyr Val Lys Arg Leu His Glu Val Gly Arg
        110                 115                 120

aca gag ccc gag ctg ctg gtg gcc cac gcc tac acc cgc tac ctg ggt      497
Thr Glu Pro Glu Leu Leu Val Ala His Ala Tyr Thr Arg Tyr Leu Gly
    125                 130                 135
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Asp Leu Ser Gly Gly Gln Val Leu Lys Lys Ile Ala Gln Lys Ala Leu
140                 145                 150                 155

gac ctg ccc agc tct ggc gag ggc ctg gcc ttc ttc acc ttc ccc aac      593
Asp Leu Pro Ser Ser Gly Glu Gly Leu Ala Phe Phe Thr Phe Pro Asn
                160                 165                 170

att gcc agt gcc acc aag ttc aag cag ctc tac cgc tcc cgc atg aac      641
Ile Ala Ser Ala Thr Lys Phe Lys Gln Leu Tyr Arg Ser Arg Met Asn
            175                 180                 185

tcc ctg gag atg act ccc gca gtc agg cag agg gtg ata gaa gag gcc      689
Ser Leu Glu Met Thr Pro Ala Val Arg Gln Arg Val Ile Glu Glu Ala
        190                 195                 200

aag act gcg ttc ctg ctc aac atc cag ctc ttt gag gag ttg cag gag      737
Lys Thr Ala Phe Leu Leu Asn Ile Gln Leu Phe Glu Glu Leu Gln Glu
    205                 210                 215

ctg ctg acc cat gac acc aag gac cag agc ccc tca cgg gca cca ggg      785
Leu Leu Thr His Asp Thr Lys Asp Gln Ser Pro Ser Arg Ala Pro Gly
220                 225                 230                 235

ctt cgc cag cgg gcc agc aac aaa gtg caa gat tct gcc ccc gtg gag      833
Leu Arg Gln Arg Ala Ser Asn Lys Val Gln Asp Ser Ala Pro Val Glu
                240                 245                 250

act ccc aga ggg aag ccc cca ctc aac acc cgc tcc cag gct ccg ctt      881
Thr Pro Arg Gly Lys Pro Pro Leu Asn Thr Arg Ser Gln Ala Pro Leu
            255                 260                 265

ctc cga tgg gtc ctt aca ctc agc ttt ctg gtg gcg aca gtt gct gta      929
Leu Arg Trp Val Leu Thr Leu Ser Phe Leu Val Ala Thr Val Ala Val
        270                 275                 280

ggg ctt tat gcc atg tgaatgcagg catgctggct cccagggcca tgaactttgt      984
Gly Leu Tyr Ala Met
    285

ccggtggaag gccttctttc tagagaggga attctcttgg ctggcttcct taccgtgggc   1044
actgaaggct ttcagggcct ccagccctct cactgtgtcc ctctctctgg aaaggaggaa   1104
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<213> Homo sapiens

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            20                  25                  30
Phe Met Arg Asn Phe Gln Lys Gly Gln Val Thr Arg Asp Gly Phe Lys
        35                  40                  45
Leu Val Met Ala Ser Leu Tyr His Ile Tyr Val Ala Leu Glu Glu Glu
    50                  55                  60
Ile Glu Arg Asn Lys Glu Ser Pro Val Phe Ala Pro Val Tyr Phe Pro
65                  70                  75                  80
```

```
Glu Glu Leu His Arg Lys Ala Ala Leu Glu Gln Asp Leu Ala Phe Trp
                 85                  90                  95
Tyr Gly Pro Arg Trp Gln Glu Val Ile Pro Tyr Thr Pro Ala Met Gln
            100                 105                 110
Arg Tyr Val Lys Arg Leu His Glu Val Gly Arg Thr Glu Pro Glu Leu
        115                 120                 125
Leu Val Ala His Ala Tyr Thr Arg Tyr Leu Gly Asp Leu Ser Gly Gly
    130                 135                 140
Gln Val Leu Lys Lys Ile Ala Gln Lys Ala Leu Asp Leu Pro Ser Ser
145                 150                 155                 160
Gly Glu Gly Leu Ala Phe Phe Thr Phe Pro Asn Ile Ala Ser Ala Thr
                165                 170                 175
Lys Phe Lys Gln Leu Tyr Arg Ser Arg Met Asn Ser Leu Glu Met Thr
            180                 185                 190
Pro Ala Val Arg Gln Arg Val Ile Glu Glu Ala Lys Thr Ala Phe Leu
        195                 200                 205
Leu Asn Ile Gln Leu Phe Glu Glu Leu Gln Glu Leu Leu Thr His Asp
    210                 215                 220
Thr Lys Asp Gln Ser Pro Ser Arg Ala Pro Gly Leu Arg Gln Arg Ala
225                 230                 235                 240
Ser Asn Lys Val Gln Asp Ser Ala Pro Val Glu Thr Pro Arg Gly Lys
                245                 250                 255
Pro Pro Leu Asn Thr Arg Ser Gln Ala Pro Leu Leu Arg Trp Val Leu
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                           Met Ser Ala Glu Val Glu Thr Ser Glu
                             1               5

ggg gta gac gag tca gaa aaa aag aac tct ggg gcc cta gaa aag gag         159
Gly Val Asp Glu Ser Glu Lys Lys Asn Ser Gly Ala Leu Glu Lys Glu
 10                  15                  20                  25

aac caa atg aga atg gct gac ctc tca gag ctc ctg aag gaa ggg acc         207
Asn Gln Met Arg Met Ala Asp Leu Ser Glu Leu Leu Lys Glu Gly Thr
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aag gaa gca cac gac cgg gca gaa aac acc cag ttt gtc aag gac ttc         255
Lys Glu Ala His Asp Arg Ala Glu Asn Thr Gln Phe Val Lys Asp Phe
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ttg aaa ggc aac att aag aag gag ctg ttt aag ctg gcc acc acg gca         303
Leu Lys Gly Asn Ile Lys Lys Glu Leu Phe Lys Leu Ala Thr Thr Ala
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ctt tac ttc aca tac tca gcc ctc gag gag gaa atg gag cgc aac aag         351
Leu Tyr Phe Thr Tyr Ser Ala Leu Glu Glu Glu Met Glu Arg Asn Lys
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gac cat cca gcc ttt gcc cct ttg tac ttc ccc atg gag ctg cac cgg         399
Asp His Pro Ala Phe Ala Pro Leu Tyr Phe Pro Met Glu Leu His Arg
 90                  95                  100                 105
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Lys Glu Ala Leu Thr Lys Asp Met Glu Tyr Phe Phe Gly Glu Asn Trp
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gag gag cag gtg cag tgc ccc aag gct gcc cag aag tac gtg gag cgg      495
Glu Glu Gln Val Gln Cys Pro Lys Ala Ala Gln Lys Tyr Val Glu Arg
            125                 130                 135

atc cac tac ata ggg cag aac gag ccg gag cta ctg gtg gcc cat gca      543
Ile His Tyr Ile Gly Gln Asn Glu Pro Glu Leu Leu Val Ala His Ala
        140                 145                 150

tac acc cgc tac atg ggg gat ctc tcg ggg ggc cag gtg ctg aag aag      591
Tyr Thr Arg Tyr Met Gly Asp Leu Ser Gly Gly Gln Val Leu Lys Lys
    155                 160                 165

gtg gcc cag cga gca ctg aaa ctc ccc agc aca ggg gaa ggg acc cag      639
Val Ala Gln Arg Ala Leu Lys Leu Pro Ser Thr Gly Glu Gly Thr Gln
170                 175                 180                 185

ttc tac ctg ttt gag aat gtg gac aat gcc cag cag ttc aag cag ctc      687
Phe Tyr Leu Phe Glu Asn Val Asp Asn Ala Gln Gln Phe Lys Gln Leu
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tac cgg gcc agg atg aac gcc ctg gac ctg aac atg aag acc aaa gag      735
Tyr Arg Ala Arg Met Asn Ala Leu Asp Leu Asn Met Lys Thr Lys Glu
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agg atc gtg gag gcc aac aag gct ttt gag tat aac atg cag ata ttc      783
Arg Ile Val Glu Ala Asn Lys Ala Phe Glu Tyr Asn Met Gln Ile Phe
        220                 225                 230

aat gaa ctg gac cag gcc ggc tcc aca ctg gcc aga gag acc ttg gag      831
Asn Glu Leu Asp Gln Ala Gly Ser Thr Leu Ala Arg Glu Thr Leu Glu
    235                 240                 245

gat ggg ttc cct gta cac gat ggg aaa gga gac atg cgt aaa tgc cct      879
Asp Gly Phe Pro Val His Asp Gly Lys Gly Asp Met Arg Lys Cys Pro
250                 255                 260                 265

ttc tac gct gct gaa caa gac aaa ggg ctg gag ggc agc ctg tcc ctt      927
Phe Tyr Ala Ala Glu Gln Asp Lys Gly Leu Glu Gly Ser Leu Ser Leu
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Pro Thr Ser Tyr Ala Val Leu Arg Lys Pro Ser Leu Gln Phe Ile Leu
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gcc gct ggt gtg gcc cta gct gct gga ctc ttg gcc tgg tac tac atg     1023
Ala Ala Gly Val Ala Leu Ala Ala Gly Leu Leu Ala Trp Tyr Tyr Met
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Glu Asn Thr Gln Phe Val Lys Asp Phe Leu Lys Gly Asn Ile Lys Lys
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Glu Leu Phe Lys Leu Ala Thr Thr Ala Leu Tyr Phe Thr Tyr Ser Ala
65                  70                  75                  80
Leu Glu Glu Glu Met Glu Arg Asn Lys Asp His Pro Ala Phe Ala Pro
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Leu Tyr Phe Pro Met Glu Leu His Arg Lys Glu Ala Leu Thr Lys Asp
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Met Glu Tyr Phe Phe Gly Glu Asn Trp Glu Glu Gln Val Gln Cys Pro
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Lys Ala Ala Gln Lys Tyr Val Glu Arg Ile His Tyr Ile Gly Gln Asn
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145                 150                 155                 160
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Leu Pro Ser Thr Gly Glu Gly Thr Gln Phe Tyr Leu Phe Glu Asn Val
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Asp Asn Ala Gln Gln Phe Lys Gln Leu Tyr Arg Ala Arg Met Asn Ala
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Ala Phe Glu Tyr Asn Met Gln Ile Phe Asn Glu Leu Asp Gln Ala Gly
225                 230                 235                 240
Ser Thr Leu Ala Arg Glu Thr Leu Glu Asp Gly Phe Pro Val His Asp
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Gly Lys Gly Asp Met Arg Lys Cys Pro Phe Tyr Ala Ala Glu Gln Asp
            260                 265                 270
Lys Gly Leu Glu Gly Ser Leu Ser Leu Pro Thr Ser Tyr Ala Val Leu
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tgaatgacca gagtatgggc agcacagaca actcaacaaa a atg tct tca gag gtg   1076
                                              Met Ser Ser Glu Val
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gag act gcg gag gcc gta gat gag tca gag aag aac tct atg gca tca     1124
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Glu Lys Glu Asn His Ser Lys Ile Ala Asp Phe Ser Asp Leu Leu Lys
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gaa ggg aca aag gaa gca gat gac cgg gca gaa aat acc cag ttt gtc     1220
Glu Gly Thr Lys Glu Ala Asp Asp Arg Ala Glu Asn Thr Gln Phe Val
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aaa gac ttc ttg aaa gga aac att aag aag gag cta ttt aag ctg gcc     1268
Lys Asp Phe Leu Lys Gly Asn Ile Lys Lys Glu Leu Phe Lys Leu Ala
      55                  60                  65

acc act gca ctt tca tac tca gcc cct gag gag gaa atg gat tca ctg     1316
Thr Thr Ala Leu Ser Tyr Ser Ala Pro Glu Glu Glu Met Asp Ser Leu
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acc aag gac atg gag tac ttc ttt ggt gaa aac tgg gag gaa aaa gtg     1364
Thr Lys Asp Met Glu Tyr Phe Phe Gly Glu Asn Trp Glu Glu Lys Val
                  90                  95                 100

aag tgc tct gaa gct gcc cag acg tat gtg gat cag att cac tat gta     1412
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              105                 110                 115

ggg caa aat gag cca gag cat ctg gtg gcc cat act tac tct act tac     1460
Gly Gln Asn Glu Pro Glu His Leu Val Ala His Thr Tyr Ser Thr Tyr
          120                 125                 130

atg ggg gga aac ctt tca ggg gac cag gta ctg aag aag gag acc cag     1508
Met Gly Gly Asn Leu Ser Gly Asp Gln Val Leu Lys Lys Glu Thr Gln
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ccg gtc ccc ttc act agg gaa ggg act cag ttc tac ctg ttt gag cat     1556
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gcc ttg gac ctg aat ttg aag acc aaa gag agg att gtg gag gaa gcc     1652
Ala Leu Asp Leu Asn Leu Lys Thr Lys Glu Arg Ile Val Glu Glu Ala
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acc aaa gcc ttt gaa tat aat atg cag ata ttc agt gaa ctg gac cag     1700
Thr Lys Ala Phe Glu Tyr Asn Met Gln Ile Phe Ser Glu Leu Asp Gln
          200                 205                 210

gca ggc tcc ata cca gta aga gaa acc cta aag aat ggg ctc tca ata     1748
Ala Gly Ser Ile Pro Val Arg Glu Thr Leu Lys Asn Gly Leu Ser Ile
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ctt gat ggg aag gga ggt gta tgc aaa tgt ccc ttt aat gct gct cag     1796
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atg gcc ttg ctg agg aag cct aac ttg cag ctc att cta gtt gcc agt      1892
Met Ala Leu Leu Arg Lys Pro Asn Leu Gln Leu Ile Leu Val Ala Ser
            265                 270                 275

atg gcc ttg gta gct gga ctt tta gcc tgg tac tac atg tgaagggcct      1941
Met Ala Leu Val Ala Gly Leu Leu Ala Trp Tyr Tyr Met
        280                 285                 290

gtcaagttgt ttgcatccta tctcaacatc ctaccacttg ttccttcccc acctccacct   2001
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caataaaaga cagaccctag aaaaaagtca tgacttaagt ggcacgggga cacctaaagt   2121
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Asn Thr Gln Phe Val Lys Asp Phe Leu Lys Gly Asn Ile Lys Lys Glu
    50                  55                  60
Leu Phe Lys Leu Ala Thr Thr Ala Leu Ser Tyr Ser Ala Pro Glu Glu
65                  70                  75                  80
Glu Met Asp Ser Leu Thr Lys Asp Met Glu Tyr Phe Phe Gly Glu Asn
                85                  90                  95
Trp Glu Glu Lys Val Lys Cys Ser Glu Ala Ala Gln Thr Tyr Val Asp
            100                 105                 110
Gln Ile His Tyr Val Gly Gln Asn Glu Pro Glu His Leu Val Ala His
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Thr Tyr Ser Thr Tyr Met Gly Gly Asn Leu Ser Gly Asp Gln Val Leu
    130                 135                 140
Lys Lys Glu Thr Gln Pro Val Pro Phe Thr Arg Glu Gly Thr Gln Phe
145                 150                 155                 160
Tyr Leu Phe Glu His Val Asp Asn Ala Lys Gln Phe Lys Leu Phe Tyr
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Cys Ala Arg Leu Asn Ala Leu Asp Leu Asn Leu Lys Thr Lys Glu Arg
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Ile Val Glu Glu Ala Thr Lys Ala Phe Glu Tyr Asn Met Gln Ile Phe
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225                 230                 235                 240
Phe Asn Ala Ala Gln Pro Asp Lys Gly Thr Leu Gly Gly Ser Asn Cys
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Pro Phe Gln Met Ser Met Ala Leu Leu Arg Lys Pro Asn Leu Gln Leu
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Ile Leu Val Ala Ser Met Ala Leu Val Ala Gly Leu Leu Ala Trp Tyr
        275                 280                 285
Tyr Met
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/19823

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/44 A61K48/00 A61P9/10 //A61K38/18,A61K31/555

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABRAHAM, N. G. (1) ET AL: "Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: Protective effect against heme and hemoglobin toxicity." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1995) VOL. 92, NO. 15, PP. 6798-6802. , XP002100374 the whole document ---	1-12
X	WO 98 08566 A (WISCONSIN MED COLLEGE INC ;UNIV DUKE (US)) 5 March 1998 (1998-03-05) page 4, line 23 -page 6, line 12 page 7, line 24 -page 8, line 2 page 9, line 19 -page 10, line 18 page 55, line 2 - line 30 page 57, line 22 - line 28 ---	14-25

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2000	09/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Stein, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 36615 A (HARVARD COLLEGE) 9 October 1997 (1997-10-09) page 2, line 11 - line 36 page 7, line 16 -page 8, line 13 page 16, line 18 -page 17, line 36 page 21, line 14 -page 22, line 8 claims 1-6	14-23
A	MAULIK N ET AL: "Nitric oxide/carbon monoxide. A molecular switch for myocardial preservation during ischemia." CIRCULATION, (1996 NOV 1) 94 (9 SUPPL) II398-406. , XP000876907 the whole document	1-13
A	ABRAHAM, NADER G. (1): "Manipulation of heme oxygenase expression by gene transfer and metals: Implications in cell injury and repair." JOURNAL OF NEUROCHEMISTRY, (1998) VOL. 70, NO. SUPPL. 1, PP. S45. MEETING INFO.: 29TH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR NEUROCHEMISTRY DENVER, COLORADO, USA MARCH 7-11, 1998 AMERICAN SOCIETY FOR NEUROCHEMISTRY. , XP000876935 the whole document	1-13
A	LONG, XILIN ET AL: "Hypoxia-induced expression of heme oxygenase gene expression in cultured neonatal rat cardiac myocytes." CIRCULATION, (1995) VOL. 92, NO. 8 SUPPL., PP. I653-I654, XP000876926 the whole document	1-13
A	HOSHIDA, SHIRO ET AL: "Heme oxygenase -1 as a culture shock protein in rat neonatal cardiomyocytes." JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, (1994) VOL. 26, NO. 11, PP. CCXII, XP000876927 the whole document	1-13
A	BORGER DR: "Induction of HSP 32 gene in hypoxic cardiomyocytes is attenuated by treatment with N-acetyl-L-cysteine" AMERICAN JOURNAL OF PHYSIOLOGY, vol. 274, no. 3 Pt 2, March 1998 (1998-03), pages H965-73, XP002131421 the whole document	1-14

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MORITA T ET AL: "Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells" JOURNAL OF BIOLOGICAL CHEMISTRY, (26 DEC 1997) VOL. 272, NO. 52, PP. 32804-32809, XP002131422 the whole document ---	24,25
P,X	SOARES M P ET AL: "Expression of heme oxygenase -1 can determine cardiac xenograft survival." NATURE MEDICINE, (1998 SEP) 4 (9) 1073-7., XP002131423 the whole document -----	13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 19823

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-8, 14-25 are directed to a method of treatment of the human/animal the search has been carried out and based based on the alleged effects of the compound /composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/19823

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9808566 A	05-03-1998	AU 4054297 A	19-03-1998
		EP 0963219 A	15-12-1999
WO 9736615 A	09-10-1997	US 5888982 A	30-03-1999